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The Role of Janus-Kinase-3 in CD4$^+$ T Cell Proliferation and Differentiation: A Dissertation

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THE ROLE OF JANUS-KINASE-3 IN CD4^{+} T CELL PROLIFERATION AND DIFFERENTIATION

A Dissertation Presented

By

Min Shi

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IMMUNOLOGY AND VIROLOGY PROGRAM

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ABSTRACT

Jak3, a member of the Janus family of tyrosine kinases, is essential for signaling via the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These Jak3-dependent cytokines primarily activate STAT5 and are critical for lymphoid generation and differentiation. Using naïve $CD4^+$ T cells from Jak3-deficient mice and wild type $CD4^+$ T cells treated with a pharmacological inhibitor of Jak3, we report that Jak3-dependent cytokine signals are not required for the proliferation of naïve $CD4^+$ T cells. This is illustrated by the similar percentage of divided cells, comparable cell divisions, intact cell cycle progression and unaffected regulation of cell cycle proteins in the absence of Jak3. In contrast to proliferation, differentiation of naïve $CD4^+$ T cells into Th1 effector cells requires Jak3-dependent cytokine signals. In the absence of Jak3, naïve $CD4^+$ T cells proliferate robustly, but produce little IFN-γ after Th1 polarization *in vitro*. This defect is not due to reduced activation of STAT1 or STAT4, nor to impaired up-regulation of the transcription factor T-bet. Instead, we find that T-bet binding to the *Ifng* promoter is greatly diminished in the absence of Jak3-dependent signals, correlating with a decrease in *Ifng* promoter accessibility and histone acetylation. These data indicate that while Jak3-dependent signals are dispensable for naïve $CD4^+$ T cell proliferation, Jak3 regulates epigenetic modification and chromatin remodeling of the *Ifng* locus during Th1 differentiation.

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CHAPTER I

Introduction

The immune system

The mammalian body is susceptible to infection by a wide range of pathogens, such as viruses, bacteria, fungi and parasites. To effectively defend against infection, the host forms both innate and adaptive immune responses to remove pathogenic microorganisms. The innate immune mechanisms act as the front line of host defense to protect the individual from invading microorganisms and to develop an immediate reaction after pathogen exposure. If pathogens elude or overcome innate defense mechanisms, adaptive immunity is triggered with the generation of antigen-specific effector cells, which target the specific infectious agents and have the potential to develop into long-lasting memory cells that prevent reinfection with the same microorganisms. The crucial first step in the adaptive immune response is the activation of naïve antigen-specific T cells.

Following development in the thymus, naïve T cells enter the bloodstream and circulate between blood and peripheral lymphoid tissue. When naïve T cells meet foreign peptides bound to self MHC molecules presented by antigen presenting cells, they become activated, and subsequently proliferate and differentiate into armed effector cells, including T helper (Th) CD4 cells and cytotoxic CD8 T cells. These effector cells play an essential role in cell-mediated and humoral immune responses to pathogens. Cell-mediated immune responses involve the destruction of intracellular pathogens by macrophages, which are activated by Th cells, and the destruction of infected cells by cytotoxic T cells. Humoral responses are initiated by Th cells through activating specific

naïve B cells to differentiate into plasma cells, which are capable of making antibodies to clear infectious agents.

Given the essential role of lymphocytes in immune responses, the regulation of these cells throughout their lifetime is necessary. Failure of regulation results in persistent diseases or unwanted immune responses, such as autoimmune disease, transplant rejection and allergy. To minimize the occurrence of these consequences, multiple steps of lymphocyte regulation exist, which begin with developmental regulation and continue with functional regulation. These regulations are carried out by different mechanisms that are mediated by cell surface receptors, intracellular signaling molecules and soluble factors.

Cytokines are one of the most important soluble factors that participate in the regulation of the development and function of lymphocytes to establish proper immunity responses. Cytokines are small proteins that are released by various cells in the body in response to stimulus and can affect the behavior of cells bearing receptors for them. Binding of cytokines to their specific receptors activates intracellular signaling pathways, which promote cell effector function. Cytokines can act in an autocrine manner (affecting the cells which release the cytokines), in a paracrine manner (affecting the adjacent cells), or in an endocrine manner (affecting the distant cells). The common γ chain (γc) cytokine family expresses the γc on the cell surface and uses Janus kinase 3 (Jak3) intracellularly. This family of cytokines is critical for lymphocyte development, proliferation, survival

and differentiation. The cytokines within this family include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. The overall goal of this dissertation is to further investigate the importance of γc cytokines and their master kinase Jak3 in T lymphocyte proliferation and differentiation.

T lymphocyte proliferation

Quiescent T cells stay in the G0 stage of the cell cycle. Following proper stimulation, quiescent T cells exit the G0 phase and enter into the cell cycle to undergo cell division. Cell division drives clonal expansion to generate a pool of T cells that are capable of recognizing a specific antigen. Also, cell division drives effector T cell differentiation at the single cell level, illustrated by the fact that effector cytokine expression requires cell cycle progression (1).

The cell cycle includes G1, S, G2 and M phases (Figure 1.1). The G1 phase involves significant synthesis of enzymes required for S phase. The S phase is the DNA synthesis stage, during which the amount of DNA doubles in cells. The G2 phase is marked by the synthesis of microtubule proteins that are required during the process of mitosis. The M phase is composed of nuclear division and cytoplasmic division.

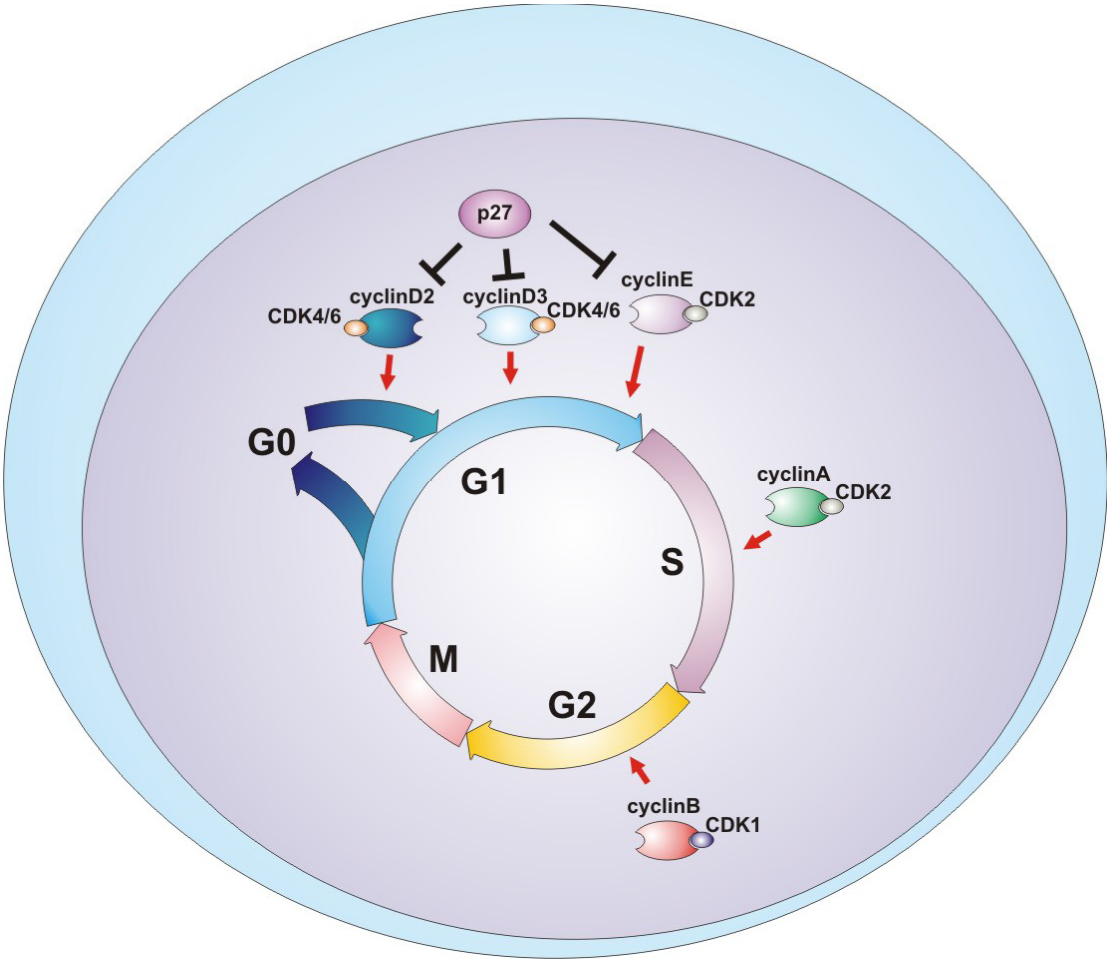
The molecular events that control the cell cycle occur in an ordered and directional sequence (Figure 1.1). The key regulatory molecules, cyclins and cyclin-dependent kinases (CDK), determine the cell cycle progression. Cyclins together with CDKs form

holoenzymes, where cyclins are regulatory subunits and CDKs are catalytic subunits. Activated by cyclins, CDKs induce specific target proteins to orchestrate the entry into the next phase of the cell cycle. CDKs are constitutively expressed in cells, whereas cyclins are synthesized at various stages of the cell cycle. Cyclin D is the first cyclin synthesized in the early G1 phase and binds to CDK4/6 to form the cyclin D/CDK complex. This complex phosphorylates retinoblastoma protein (Rb), leading to the activation of E2F. In turn, E2F promotes the transcription of varying genes, such as cyclin E, cyclin A and DNA polymerase. Cyclin E is produced at the late stage of the G1 phase and binds to the existing CDK2 to drive the transition from G1 to S phase. Substantial cyclin A synthesis occurs in the S phase, and cyclin A also binds to CDK2 to form the cyclin A/CDK2 complex to initiate entry into the G2/M phase. Cyclin B is up-regulated during the G2/M phase, and the Cyclin B/CDK1 complex causes the breakdown of the nuclear envelope and the initiation of mitosis. The extracellular events inducing cell cycle machinery consist of signals from antigen, costimulatory and growth factors. These signals will be discussed in detail in chapter two.

Figure 1.1 The scheme of the cell cycle.

The cell cycle includes G1, S, G2 and M phases. Progression of a cell cycle is promoted by a number of cyclins which, when complexed with specific CDKs, drive the cell forward through the cell cycle. Cyclin D, associating with CDK4 and CDK6, controls progression through the G1 phase of the cell cycle by phosphorylation of Rb and activation of E2F. Cyclin E/CDK2 complex activity is rate-limiting for the S phase entry. Cyclin A/CDK2 drives the transition from the S phase to the G2/M phase. Cyclin B/CDK1 complex initiates mitosis and its deactivation causes the exit of the cell cycle. p27kip1 halts the cell cycle in the G1 phase by binding to and inactivating cyclin D/CDK4/6 and cyclin E/CDK2 complexes.

Figure 1.1


p27
cyclinD2
CDK4/6
cyclinD3
CDK4/6
cyclinE
CDK2
G0
G1
S
cyclinA
CDK2
M
G2
cyclinB
CDK1

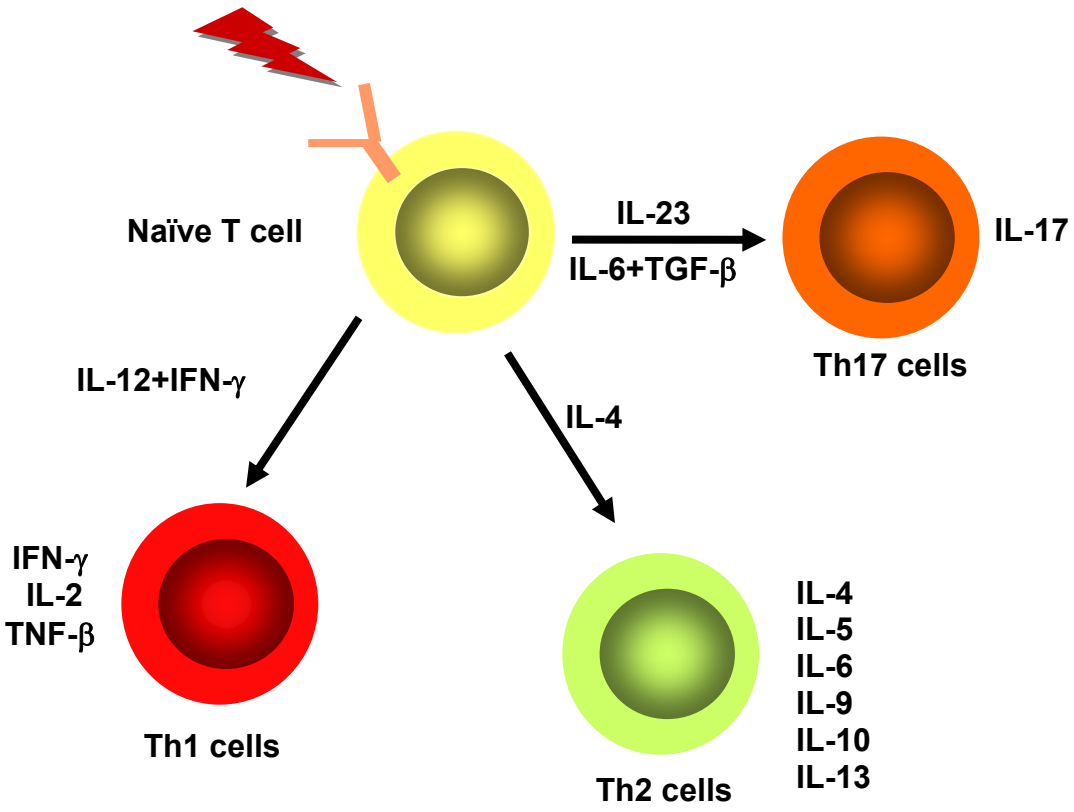
T helper cell differentiation and the extracellular regulators

T helper lymphocytes are responsible for orchestrating the appropriate immune responses to a number of pathogens. The differentiation of a T helper cell is initiated when the T cell receptor (TCR) encounters its cognate antigen bound to MHC molecules on a dendritic cell (DC). There are at least three subsets of helper T cells, which are T helper 1 (Th1), T helper 2 (Th2) and the newly discovered T helper 17 (Th17) cells (Figure 1.2). Th1 cells produce IFN-γ and TNF-β and are essential for the elimination of intracellular pathogens such as *Listeria monocytogenes* and *Leishmania major*. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13, which are critical for the eradiation of extracellular organisms including helminthes. Th17 cells produce IL-17 and IL-22 and are important for host defense against *Klebsiella pneumonia* and *Bacteroides fragilis*. Excess of Th1 cells, along with Th17 cells, mediate organ-specific autoimmunity, such as type I diabetes, multiple sclerosis, contact dermatitis, rheumatoid arthritis, lyme arthritis, psoriasis and inflammatory bowel disease. Excess of Th2 cells is implicated in allergy and asthma.

Figure 1.2 Development of T helper cell subsets.

Naive $CD4^+$ T cells are activated by antigen-presenting cells in the presence of co-stimulatory molecules, and differentiate into functionally distinct T helper cell subsets (Th1, Th2 or Th17) under the influence of indicated specific cytokines. The fully-differentiated T helper cell subtypes influence the immune system in part through the secretion of specific cytokines.

Figure 1.2


Naïve T cell
IL-23
IL-6+TGF-β
IL-17
Th17 cells
IL-12+IFN-γ
IL-4
IFN-γ
IL-2
TNF-β
Th1 cells
Th2 cells
IL-4
IL-5
IL-6
IL-9
IL-10
IL-13

Th differentiation is a finely balanced process, which is influenced by a lot of factors, including the type of antigen presenting cells, the duration and strength of signals, the ligation of costimulatory molecules, and most importantly, the local cytokine milieu. The identification of subsets of DCs has suggested that distinct subsets of DCs are involved in Th fate determination (2, 3). For instance, in the mouse system, CD8α+ DCs preferentially stimulate Th1 cell differentiation by secreting IL-12, whereas CD8α- DCs promote Th2 cell differentiation most likely through the production of IL-6. In addition to the type of DCs, the antigen dose can influence Th polarization (4). Several *in vitro* and *in vivo* studies have demonstrated that high doses of antigen are required for Th1 cell generation. These studies demonstrate that strong TCR/peptide interaction and sustained signal transduction are necessary for proliferative responses and IFN-γ production. When priming with low doses of antigen, only a transient signaling is generated and results in Th2 cell differentiation (4). Further, costimulatory molecules are involved in Th development. CD28/B7 (5), ICOS/B7RP-1 (6) and CD28-dependent OX40 signals (7) are important for generating a Th2 cell response, and B7-H3 is critical for Th1 differentiation (8).

Cytokines play an essential role in T helper cell differentiation. Cytokines stimulate helper T cell maturation through inducing one lineage-specific transcription factor while suppressing others to select for a specific lineage. IL-12, IL-18, IFN-γ and IL-27 significantly influence Th1 cell polarization, whereas IL-4 and IL-2 remain the eminent

cytokines for Th2 cell development. IL-23, IL-6, IL-21 and TGF-β are involved in the differentiation of naïve $CD4^+$ T cells into Th17 cells.

IL-12 is produced mainly by activated macrophages and DCs and is a heterodimer composed of two subunits, p35 and p40. Naïve $CD4^+$ T cells are unresponsive to IL-12 due to lack of IL-12 receptor (IL-12R) β2 expression, which is required to form a functional IL-12R with IL-12R β1 chain. Both IL-12R β1 and IL-12R β2 are up-regulated by activation through the TCR. However, IL-12R β2 is expressed in Th1 differentiating cells and extinguished in Th2 differentiating cells, which serves as a mechanism by which CD4 T cells are able to commit to a particular Th cell lineage. Binding of IL-12 to IL-12R activates the Janus family tyrosine kinase (Jak) and signal transducer and activator of transcription (STAT) signaling pathway, specifically the Jak2 and Tyk2 kinases and STAT4. Mice deficient in IL-12, IL-12R β1, IL-12R β2 and STAT4 have profoundly reduced Th1 responses, indicating the central role of IL-12 signaling in Th1 immunity. Although IL-12/STAT4 signaling is not needed for initial Th1 cell differentiation, it amplifies Th1 cell responses by augmenting the production of IFN-γ and promoting the expression of the IL-18 receptor (IL-18R) in differentiating Th1 cells. IL-18 is a member of the IL-1 family of cytokines and is secreted by activated macrophages and DCs. It acts synergistically with IL-12 to enhance IFN-γ production (9). Besides being the signature cytokine of Th1 cells, IFN-γ is a cytokine that educates naïve $CD4^+$ T cells to differentiate into Th1 cells. In addition to naïve $CD4^+$ T cells and Th1 cells, IFN-γ is produced by nature killer (NK) cells, $CD8^+$ T cells, macrophages and DCs.

IFN-γ promotes Th1 cell differentiation through binding to its receptor, which is composed of IFN-γ receptor (IFN-γR) 1 and IFN-γR2. The IFN-γR is present on many cell types and utilizes Jak1 and Jak2 kinases and the transcription factor STAT1. IFN-γ activates and reinforces the production of IL-12 by macrophages and DCs to indirectly induce Th1 cell polarization. In addition, IFN-γ can directly induce T cells to differentiate into Th1 cells by promoting the expression of the Th1 cell master transcription factor, T-bet. Mice deficient in IFN-γ, IFN-γR or STAT1 have impaired immune responses to *L. major*, which is controlled via the development of a Th1 cell response. IL-27 is also important in Th1 cell differentiation. IL-27 is a novel cytokine that is structurally related to the IL-12 family and is mainly produced by activated macrophages. It signals through the IL-27 receptor (IL-27R), which is expressed in naïve $CD4^+$ T cells and NK cells. Although IL-27 is thought to promote inflammation by inducing Th1 cell differentiation (10, 11), recent studies demonstrate that IL-27, together with IL-6 and TGF-β, promotes IL-10 production by activated $CD4^+$ T cells to suppress pro-inflammatory immune responses (12-15).

IL-4 is critical for Th2 cell differentiation and potentially initiates the development of these cells. IL-4 is secreted by many cell types, such as Th2 cells, naïve $CD4^+$ T cells, mast cells, basophils, eosinophils and natural killer T (NKT) cells. IL-4 functions through the IL-4 receptor (IL-4R), which is expressed on naïve $CD4^+$ T cells and consists of the common γ chain (γc) subunit and the IL-4Rα chain. After binding of IL-4 to IL-4R, Jak1 and Jak3 kinases are recruited to the receptor complex to activate STAT6. This signaling

event mediates the induction of the master transcription factor for Th2 cells, GATA3. In addition, IL-2 has been implicated in Th2 cell differentiation (16). IL-2 signals through the IL-2 receptor (IL-2R), which is composed of IL-2Rα, IL-2Rβ and the γc chain. IL-2Rβ and the γc chain are expressed on the surface of naïve $CD4^+$ T cells, whereas the expression of IL-2Rα is induced after TCR engagement. Jak1 and Jak3 are downstream of the IL-2R complex and promote STAT5 activation. The effect of IL-2 on Th2 cell differentiation is through the activation of STAT5, which will be discussed in the later section.

It has previously been thought that IL-23 is involved in Th1 cell differentiation (17). However, recent studies showed that IL-23 is closely associated with a novel subset of pro-inflammatory $CD4^+$ T cells that produce IL-17 (18)**.** IL-23 is another member of the IL-12 family and is a heterodimer composed of the p40 subunit of IL-12 and a novel subunit, p19. The receptor for IL-23 has two subunits. One subunit shares the IL-12Rβ1 with IL-12 and the second subunit is IL-23R. The Jak/STAT pathway utilized in IL-23 signaling includes Jak2, Tyk2, STAT1 and STAT3. Naïve $CD4^+$ T cells do not express the IL-23R, therefore, IL-23 appears to not be responsible for the initial induction or commitment of Th17 cell. Rather, IL-23 is essential for the maintenance or expansion of Th17 cells. *In vitro* data show that the differentiation of Th17 cells is induced by the combination of IL-6 and TGF-β (19). IL-6 acts on naïve T cells to promote the expression of IL-21, which promotes continued expression of IL-21 through an autocrine pathway (20, 21). In addition to the inhibition of IFN-γ and IL-4-dependent pathways,

TGF-β synergizes with IL-21 to induce the expression of retinoic acid receptor-related orphan receptor γ-t (RORγt), a master transcription factor for Th17 cells, via a STAT3-dependent mechanism (21, 22).

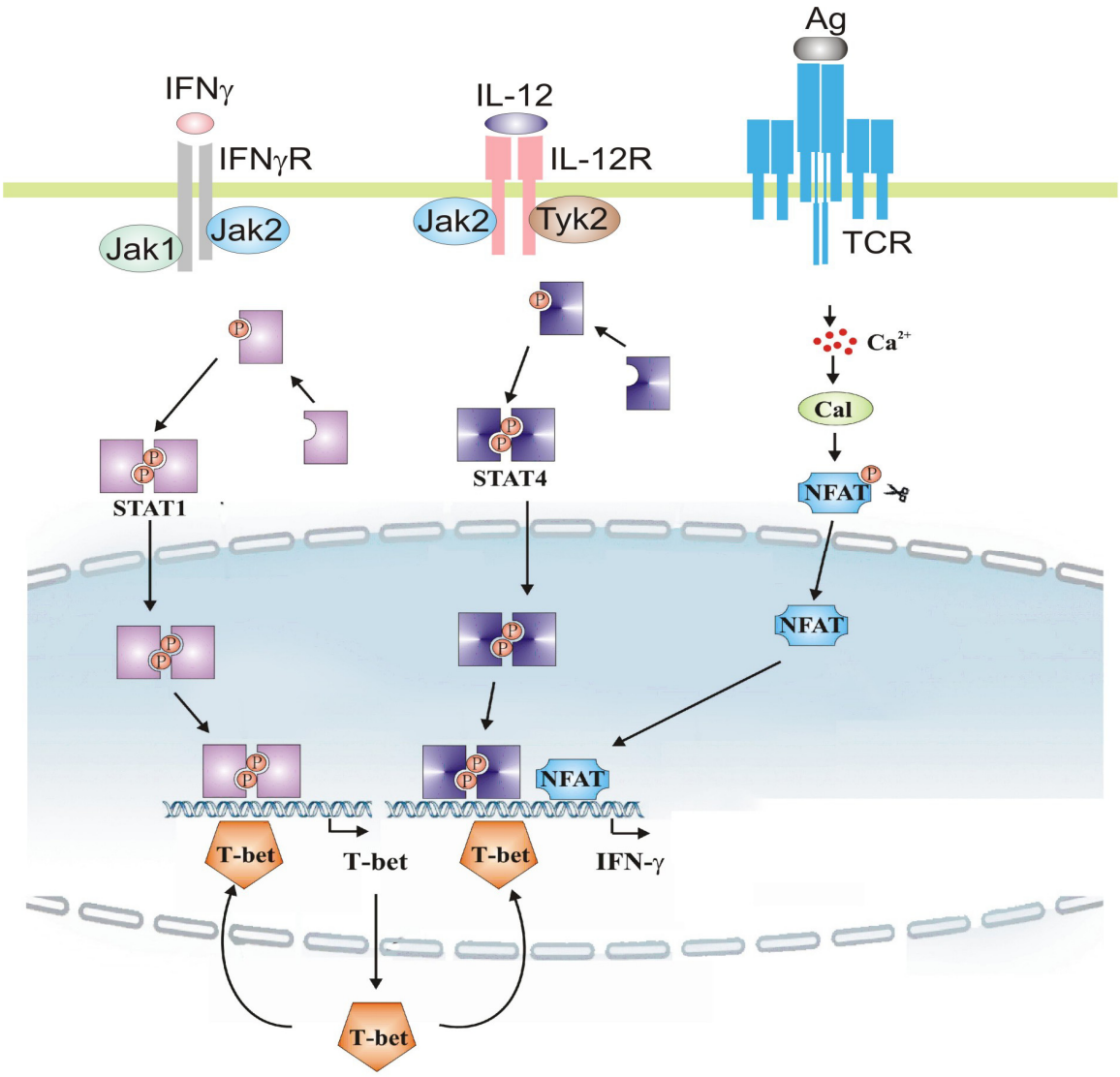
Signaling pathways and transcription factors in Th1 cell differentiation

CD4 helper T cells are classified by their distinct patterns of cytokine gene expression, which is controlled at the level of transcription. Understanding the signaling pathways and transcriptional mechanisms that determine the expression of IFN-γ and IL-4 has led to insights into the regulation of lineage commitment of T helper cells. Over the past twenty years, tremendous progress has been made in identifying the signaling pathways and transcription factors that control Th1 (Figure 1.3) and Th2 cells.

Figure 1.3 Signaling pathways in Th1 differentiation.

Signals from TCR ligation trigger the release of Ca2+ from the ER, which promotes the entry of extracellular Ca2+ into cells. Calcium-bound calmodulin activates the phosphatase calcineurin (Cal), which dephosphorylates NFAT. Dephosphorylated NFAT goes into nucleus and non-selectively stimulates both IFN-γ and IL-4 production. After IFN-γ binds to the IFN-γ receptor (IFN-γR), Jak1 and Jak2 are activated which phosphorylate STAT1. Phosphorylated STAT1 translocates into nucleus to promote the expression of the most important Th1 transcription factor, T-bet. T-bet, in turn, increases IFN-γ and IL-12Rβ2 expression, inhibits IL-4 expression, and maintains Th1-cell-lineage commitment. In the mean time, IL-12R signaling activates STAT4 through Jak2 and Tyk2, which further enhances the production of IFN-γ.

Figure 1.3


Ag
IFNγ
IL-12
IFNγR
IL-12R
Jak1
Jak2
Jak2
Tyk2
TCR
Ca2+
Cal
NFAT
STAT1
STAT4
NFAT
NFAT
T-bet
T-bet
T-bet
IFN-γ
T-bet

T-bet

An important advance in searching for transcription factors that underlie the cellular program of Th1 cell differentiation was the discovery of T-bet (T-box expressed in T cells). T-bet, also known as Tbx21, belongs to the T-box family of transcription factors, which have homology within a 200-amino acid DNA-binding domain called the T-box. T-bet is expressed exclusively in the lymphoid tissues, especially in the spleen, thymus, lymph node and lung. T-bet is detected in a variety of cells that produce IFN-γ, including $CD4^+$ T cells, $CD8^+$ T cells and NK cells. In $CD4^+$ T cells, T-bet is rapidly and specifically induced in developing Th1 but not Th2 cells, and T-bet expression correlates with induction of IFN-γ.

It was originally thought that T-bet might be regulated by the IL-12/STAT4 pathway to mediate Th1 cell differentiation. However, this theory was challenged by Reiner and colleagues via the demonstration that T-bet mRNA is normal in STAT4-deficient T cells under Th1-polarizing conditions. Further, this group show that T-bet acts prior to the IL-12-dependent effect on Th1 cell differentiation (23). In addition, T-bet expression has been shown to be controlled by the IFN-γ/STAT1 signaling pathway but not by the IL-12/STAT4 pathway (24, 25). Overexpression of T-bet in Th2 cells leads to the endogenous T-bet expression, which suggests that induction of T-bet occurs in an autocrine mechanism (26). However, evidence is lacking for a STAT1-independent mechanism of T-bet autoactivtion, and thus, STAT1 is indispensable for T-bet expression (25).

Gain-of-function and loss-of-function in T-bet demonstrate that T-bet plays a central role in Th1 cell differentiation. For example, retrovial transduction of T-bet into naïve $CD4^+$ T cells results in endogenous IFN-γ production (27). Overexpression of T-bet in developing or fully differentiated Th2 cells leads to a dramatic increase in IFN-γ production and a decrease in IL-4 secretion, indicating the powerful function of T-bet in redirecting Th2 cells at various stages into Th1 subset (27). The essential role of T-bet in regulating Th1 cells is confirmed by analysis of mice lacking T-bet. T-bet-deficient mice have no abnormality in thymocyte development or peripheral lymphoid organ homing (28). *In vitro*, isolated T-bet-deficient $CD4^+$ T cells have a severe defect in IFN-γ production in response to Th1-inducing conditions. Further, mice lacking T-bet fail to establish a Th1 cell response to *L.major* infection *in vivo*. Moreover, T-bet-deficient mice develop a spontaneous reactive airway disease similar to human asthma (29). Taken together, T-bet is essential in initiating Th1 cell development from naïve $CD4^+$ T cells.

The mechanisms of T-bet on Th1 cell differentiation are not clear. Some studies suggest that the function of T-bet in promoting Th1 cell development may be indirect. For instance, overexpression of T-bet promotes the expression of IL-12R β2 chain, leading to the responsiveness of $CD4^+$ T cells to IL-12 and subsequently enhancing Th1 cell polarization (25). Further, in T-bet-deficient mice, the failure to generate a Th1 cell response is always accompanied by an increase of a Th2 cell response, suggesting the critical role of T-bet in Th1 cell differentiation may partially be through repressing the

Th2 cell program. On the other hand, some evidence showed that T-bet directly regulates IFN-γ production. Several putative T-box binding sites were identified in the *Ifng* gene locus (30). T-bet either is capable of potently transactivating the *Ifng* gene (27, 31) or plays a role in chromatin remodeling of the *Ifng* gene, which will be discussed in detail later.

STAT4

STAT4 was cloned by Darnell and colleagues (32) and later found to be associated with IL-12 signaling (33). Jak2 and Tyk2 are the kinases activated by IL-12 signaling to phosphorylate STAT4. The phenotype of STAT4-deficient mice demonstrates the important role of STAT4 in Th1 cell differentiation. STAT4 knockout mice have defects in cell proliferation and IFN-γ production in response to IL-12 signaling (34, 35), and the expression of IL-18R is also impaired. Although the activation of STAT4 is correlated with the capacity of IFN-γ production, IFN-γ is not totally abrogated in the absence of STAT4, suggesting a STAT4-independent mechanism of IFN-γ production (36, 37). More studies show that STAT4 is not required for the initiation of IFN-γ production (23), but this transcription factor is essential to augment IFN-γ production and to maintain Th1 cells.

The manner in which STAT4 mediates Th1 development remains to be determined, but evidence suggests that it may transactivate IFN-γ production. Hoey and colleagues found that STAT4 binds to the nonconsensus low-affinity STAT sites within both the promoter

and first intron of the *Ifng* gene to stimulate *Ifng* transcription (38). The critical role of STAT4 activation in the interferon-gamma response during viral infection may be through direct binding to the *Ifng* gene (39). The synergistic function of IL-12 and IL-18 to enhance *Ifng* mRNA expression is dependent on activation of STAT4 since activated STAT4 up-regulates the binding activity of AP-1, an IL-18-induced activator protein (40). Other evidence indicates that STAT4 contributes to the chromatin remodeling activity of the *Ifng* gene, which will be discussed later.

STAT1

STAT1 is another signal transducer and activator of transcription involved in Th1 cell polarization. Jak1 and Jak3 activate STAT1 to carry out its biological functions. Significant progress towards understanding the function of STAT1 in cellular physiology resulted from analysis of mice lacking STAT1 expression (41). These mice display no gross developmental defect, however, they are extremely susceptible to viral infection, mainly due to the IFN resistance (41). Although the role of STAT1 in Th1 cell differentiation may be mediated by direct binding to the *Ifng* promoter (42), both *in vitro* and *in vivo* studies favor that STAT1 is associated with the induction of T-bet in Th1 cell differentiation. It has been reported that T-bet expression is dependent on IFN-γR signaling with subsequent STAT1 phosphorylation (25). *In vivo* studies show a positive feedback loop of *Ifng* gene regulation by which IFN-γ/STAT1-driven T-bet expression induces further IFN-γ production in Th1 cells (24). In addition to being the principal target of the IFN-γR signaling, STAT1 is activated by IL-27 signaling to induce T-bet

expression (10, 43). Both IFN-γR and IL-27R are expressed in naïve $CD4^+$ T cells, suggesting that STAT1 plays an important role during the initial stages of Th1 cell differentiation by inducing the expression of T-bet and the subsequent IL-12Rβ2 expression.

Hlx

Hlx (H2.0-like homeobox 1) is expressed in multiple hematopoietic lineages, including $CD4^+$ T cells, $CD8^+$ T cells, NK cells and B cells (44, 45). Reiner and colleagues (26) found that Hlx is more highly expressed in Th1 cells than Th2 cells. It appears after 3 days of Th1 cell differentiation and is induced more slowly than T-bet in differentiating Th1 cells. Retroviral overexpression of T-bet into Th2 cells induces the expression of Hlx and introduction of a dominant-negative T-bet into developing Th1 cells antagonizes the activation of Hlx transcription. Thus, Hlx appears to be a target gene of T-bet.

The function of Hlx in Th1 cell differentiation was addressed by using the gain-of-function approach, since deletion of Hlx results in lethality at embryonic day 15 due to severe hypoplasia of the liver and gut. Although ectopic expression of Hlx alone does not substantially increase IFN-γ secretion, introduction of both Hlx and T-bet results in a dramatic increase of IFN-γ expression, both in the frequency and the amount of IFN-γ produced per cell. This result indicates a synergistic action between Hlx and T-bet in Th1 cells (26). Similarly, the Hlx transgenic cells display an increased number of IFN-γ producing cells in response to keyhole limpet hemocyanin (KLH) immunization or to

culturing under Th2-skewing conditions (46, 47). Further, the dominant-negative Hlx leads to a significant impairment in IFN-γ synthesis (47). Although the molecular basis for the function of Hlx is not clear, the physically interaction between Hlx and T-bet may mediate their cooperative effects on *Ifng* transcription (47).

Ets family members

Ets family of transcription factors are characterized by an evolutionarily-conserved ETS DNA binding domain which contains a core purine-rich GGAA/T motif. In general, these transcription factors interact with a multitude of co-factors to elicit gene-specific responses and drive distinct biological processes (48), (49).

ERM, an Ets family transcription factor, is the first Th1-specific transcription factor selectively induced by IL-12 signaling through STAT4 (50) ERM regulates *Ifng* transcription via a cooperative interaction with STAT4, and over-expression of ERM by retrovirus into STAT4-deficient cells does not restore normal IFN-γ production (50).

Ets-1 (E26 transformation-specific-1) is another member of the Ets family of transcription factors. Deficiency of Ets-1 severely impairs the differentiation and function of Th1 cells (51). Ets-1 knockout cells secrete much less IFN-γ under Th1-polarizing conditions and are unable to induce colitis in SCID mice, an animal model of Th1 cell-mediated disease. There are multiple mechanisms by which Ets-1 promotes the development of Th1 cells. First, T-bet expression is markedly reduced in Ets-1-deficient

Th1 cells suggesting that Ets-1 is involved in induction of T-bet. Second, Ets-1 serves as a cofactor of T-bet by binding to the proximal region of the *Ifng* promoter to enhance the activity of T-bet. Third, Ets-1 is required for the activation of STAT4 during Th1 cell differentiation (51).

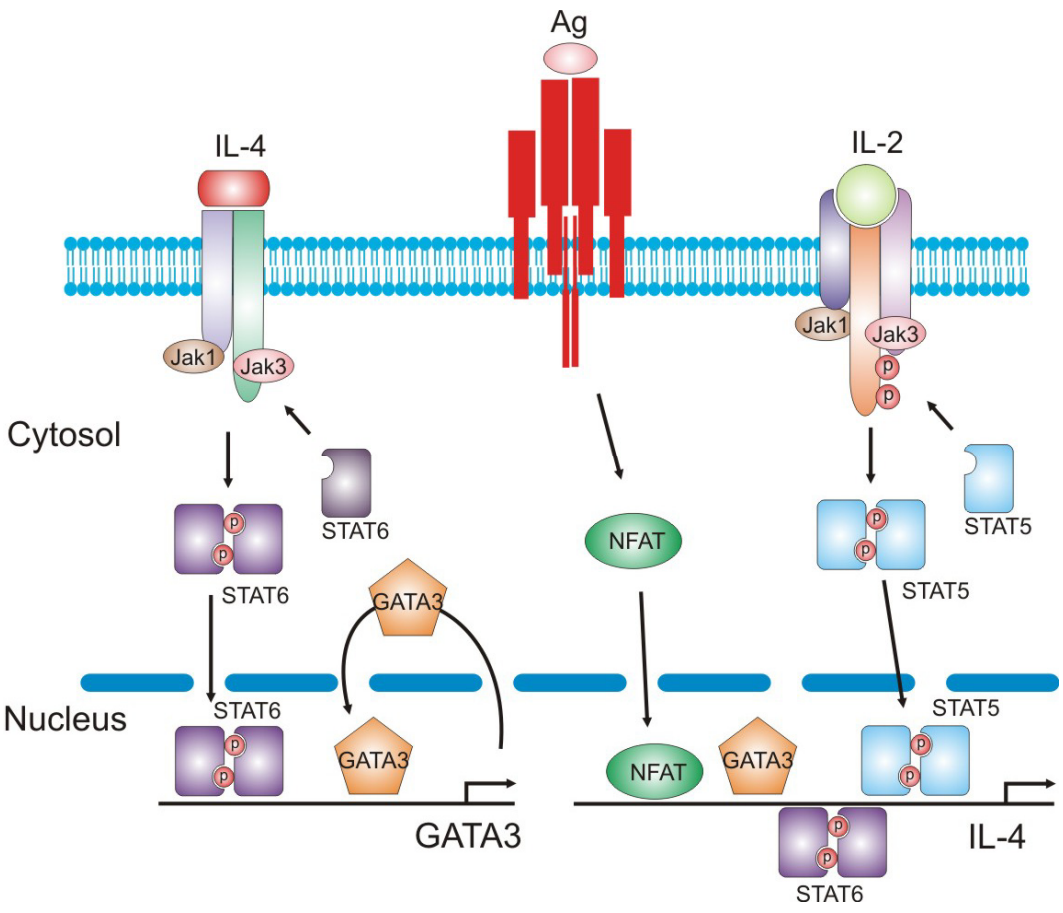
Signaling pathway and transcription factors for Th2 cell differentiation

Similar to Th1 cell differentiation, there are several transcription factors essential for Th2 cell polarization (Figure 1.4), such as GATA-3 and c-Maf. Non-Th2 cell specific factors, such as STAT6, nuclear factor of activated T cells (NFAT) and STAT5 also contribute to regulate IL-4 production. Here, I focus on GATA3, STAT6 and STAT5.

Figure 1.4 Signaling pathways in Th2 differentiation.

TCR signaling induces the activation of NFAT, a Th2 non-specific transcription factor, leading to rapid acute transcription of *Il4* gene. IL-4 receptor ligation through binding of IL-4 to IL-4R phosphorylates STAT6 via Jak1 and Jak3, resulting in the up-regulation of GATA3 expression. GATA3 expression also undergoes positive feedback by transcriptional autoactivation. The GATA3 protein in turn increases IL-4 expression, the hallmark cytokine for Th2 cells. Ligation of the IL-2R activates the associated Jak1 and Jak3 kinases, resulting in tyrosine phosphorylation of STAT5, which stabilizes the accessibility of the *Il4* gene.

Figure 1.4


Ag
IL-4
IL-2
Jak1
Jak3
Jak1
Jak3
P
P
Cytosol
STAT6
STAT6
NFAT
STAT5
STAT5
GATA3
Nucleus
STAT6
STAT5
GATA3
NFAT
GATA3
GATA3
IL-4
STAT6

STAT6

The transcription factor STAT6 is activated by Jak1 and Jak3 upon the binding of IL-4 to the IL-4R and plays a central role in modulating Th2 cell differentiation. STAT6 knockout mice have impaired Th2 cell differentiation and increased susceptibility to helminthic infections, a disease prevented by Th2 immunity (52). Ectopic expression of an inducible, activated STAT6 into developing Th1 cells significantly increases IL-4 expression (53). STAT6 function is mainly mediated by up-regulation of the Th2 cell specific transcription factor GATA3 (53), (54) or by direct binding to the *Il4* locus (55).

While STAT6 is important for maximal Th2 cell development, STAT6-deficient cells are capable of producing Th2 cell cytokines and developing *in vivo* Th2 cell responses to *S. mansoni* infection. These results suggest a STAT6-independent mechanism of differentiation of Th2 cells (56). In studies with a Th2-eliciting parasitic infection, the primary IL-4 response remains intact in STAT6-deficient mice, while secondary and memory responses are abrogated (57). These data indicate that the function of STAT6 is to stabilize rather than initiate Th2 cell development.

GATA-3

The zinc finger protein GATA-3 was originally cloned as a T-cell-specific transcription factor that binds to the enhancer of the TCRα gene (58). Although GATA-3 is very important for thymocyte development (59, 60), two groups found that the expression of GATA-3 is strongly increased during Th2 cell differentiation and decreased during Th1

cell differentiation (61, 62). GATA-3 expression is robustly up-regulated by IL-4 signaling via STAT6 (53). However, the IL-4/STAT6 pathway does not appear to be indispensible for GATA-3 induction. GATA-3 can be induced through a STAT6-independent autoactivaton pathway (63).

Introduction of GATA-3 using retroviral vectors or GATA-3 transgenic mice enhances the expression of all Th2 cell cytokines, even when $CD4^+$ T cells are cultured under Th1-skewing conditions, and a dominant-negative GATA-3 inhibits Th2 cell differentiation (61, 62). These studies suggest an essential role for GATA-3 in Th2 cell development.

The mechanisms by which GATA-3 induces Th2 cell cytokine expression depend on distinct cytokines. For instance, GATA-3 directly binds to the critical elements in the *Il5* or *Il13* promoters to induce transcription of these genes (62), (64, 65). However, the binding of GATA-3 to the promoter region of the *Il4* gene only weakly induces *Il4* transcription (66),(67). Instead, GATA-3 acts through a number of regulatory elements to control *Il4* gene expression (68). In addition to being a cis-acting transcriptional regulator, GATA-3 influences chromatin remodeling of IL-4 to affect *Il4* gene transcription (69).

STAT5

Following the binding of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 to their individual receptors, the transcription factor STAT5 is activated by Jak1 and Jak3. IL-2 has long been implicated in Th2 cell differentiation and IL-4 production (16). Recent reports

substantially clarify the role of the IL-2/STAT5 pathway in Th2 cell differentiation. In STAT5a-deficient mice, Th2 cell differentiation and $CD4^+$ T cell mediated allergic inflammation is significantly diminished (70). The intrinsic expression of STAT5a appears to be required for Th2 cell differentiation (71), and Stat5a promotes Th2 cell differentiation in a Stat6-independent manner (72). Further, it has been reported that the role of IL-2 on Th2 cell differentiation does not depend on the cell growth or survival effect of IL-2, rather, IL-2 signals transmit through STAT5 to stabilize the accessibility of the *Il4* gene (73). Overexpression of a constitutively active mutant form of STAT5a (STAT5A1*6) generates Th2 cell differentiation in the absence of IL-4 or in STAT6- or IL-4Rα-deficient cells (74). Although STAT5A1*6 does not enhance GATA-3 expression, GATA-3 synergistically acts with STAT5A1*6 to cause optimal Th2 cell priming since GATA-3 and STAT5A1*6 parallelly render the *Il4* gene accessibility (74).

Epigenetic regulation of T helper cell differentiation

"Epigenetic regulation" refers to the modification of nucleosomal histones and cytosine residues in DNA without changing the intrinsic nucleotide sequence information encoded in the DNA. Epigenetic codes are potentially heritable, to provide a means for propagating information from parental cells to their daughter cells.

Overview of epigenetic regulation of gene expression

Gene transcription in eukaryotic cells occurs in the context of chromatin, where DNA winds around a group of eight histone proteins to form a structure called a nucleosome.

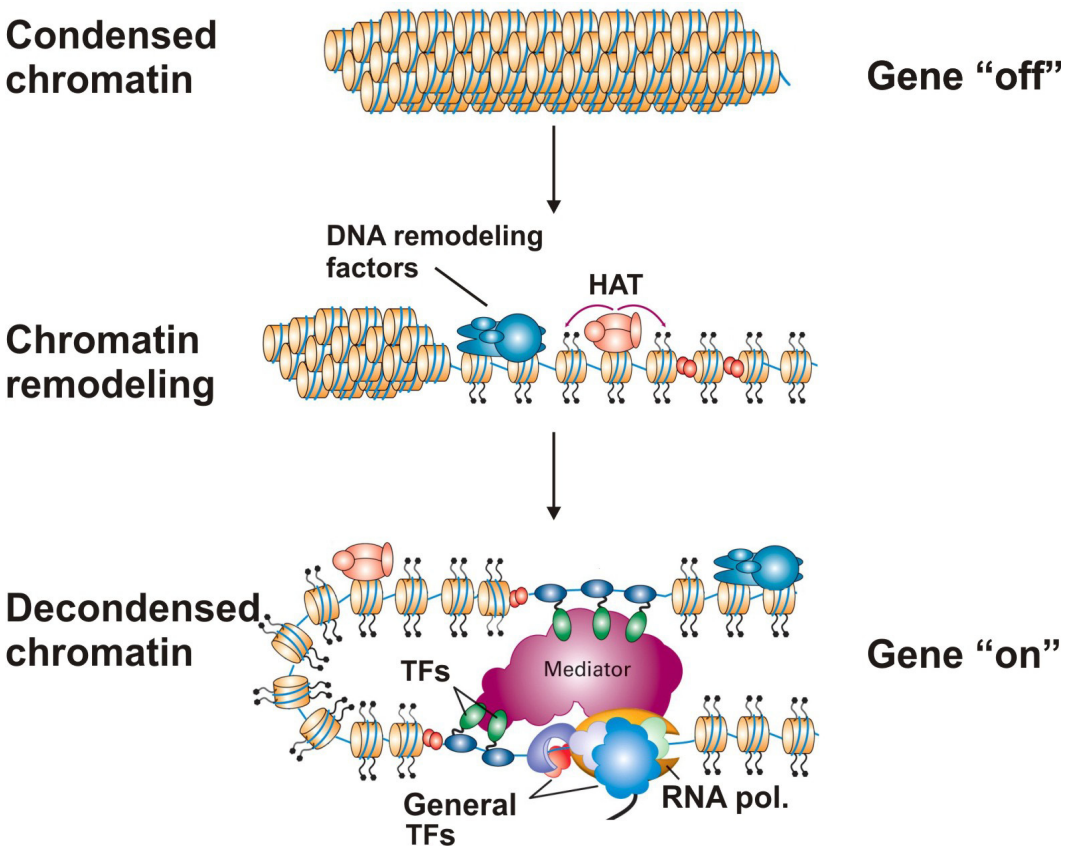
Negatively charged DNA binds to positively charged histones. Condensed chromatin generally represses gene transcription, whereas opened chromatin promotes gene expression.

In addition to differential expression of transcription factors in a cell, gene transcription is controlled by the accessibility of a gene to transcription factors. This process is mediated by binding of chromatin remodeling factors to the specific DNA element within the context of nucleosome DNA to recruit other chromatin remodeling factors, which results in the disruption of local chromatin structure and an increase in the accessibility of a gene to transcription machinery. Following chromatin remodeling, transcription factors and RNA polymerase II containing complexes are able to access the promoter sequences surrounding the coding region of a gene to start gene transcription (Figure 1.5). Thus, by modulating the accessibility of a gene to the transcription factors, epigenetic factors may facilitate or silence gene transcription.

Figure 1.5 Chromatin remodeling and gene transcription.

The condensed chromatin is generally associated with gene repression. Alteration of chromatin structure from the compact chromatin to the open chromatin is brought about by a process called chromatin remodeling, which is the first level of transcription regulation. DNA remodeling factors recruit histone acetyl transferase, which acetylates histones and opens the chromatin. Transcription factors and RNA polymerase then access the open loci and bind to their responsive elements to promote gene transcription.

Figure 1.5


Condensed chromatin
Gene "off"
DNA remodeling factors
HAT
Chromatin remodeling
Decondensed chromatin
Gene "on"
TFs
Mediator
General TFs
RNA pol.

The major mechanisms of epigenetic regulation in mammals include DNA methylation and post-translational modifications of histones. Cytosine methylation in CpG dinucleotides of DNA is strongly correlated with gene silencing (75). The amino-terminal tails of histones, as well as some internal sites, are subject to several covalent modifications, including acetylation, methylation, phosphorylation and ubiquitination. Individual histone modification can permit or repress the accessibility and transcription activity of a gene. For example, histone H3 acetylation of lysine 9 and 14 are positively associated with transcription activity; lysine 4 di- and trimethylation of histone H3 is broadly distributed throughout transcriptionally active genes. In contrast, histone H3 lysine 27 trimethylaiton and lysine 9 di- and trimethylatoin are correlated with gene silencing.

Histone acetylation has been well studied. It is proposed that hypoacetylation of histone proteins is associated with inaccessible or repressive chromatin, whereas hyperacetylation of histones is typically associated with open or active chromatin (76). The acetylation of histones is regulated by opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone hyperacetylation increases the accessibility of a gene to transcription factors via an alloseteric change in nucleosome structure, which is caused by neutralizing the charge attraction between histones and DNA (77). Some transcription factors have intrinsic HAT activity (78) and others recruit proteins which are HATs. Histone deacetylases remove the charge-neutralizing acetyl group to

deacetylate histones and silence gene expression. HDACs are usually recruited to DNA by specific transcriptional regulatory factors.

Epigenetic regulation of Th cell differentiation

The importance of epigenetic regulation in the development and proper function of the immune system is receiving more attention. Specifically, cell fate determination gives the most striking example of how epigenetic mechanisms regulate gene expression. When naïve $CD4^+$ T cells differentiate into Th1 or Th2 cells, cytokine loci undergo changes in chromatin structure. One set of genes is epigenetically activated, and the other is silenced. These alternations in chromatin structures can be heritably maintained.

IL-2 (79) and IFN-γ (80) are the first two cytokine genes whose expression are enhanced by the prevention of DNA methylation by 5-azacytidine treatment. These studies first established a correlation between changes in chromatin structure and cytokine transcription. In 1998, Rao and colleagues (81) demonstrated that T helper cell differentiation leads to long-range alterations in chromatin structure of differential cytokine genes, which gave the first definitive evidence of chromatin remodeling at cytokine gene loci being functionally associated with productive T cell differentiation. Reiner and colleagues (1) found that presence of inhibitors of histone deacetylase or cytosine methylation during T helper cell differentiation profoundly represses effector cytokine expression, confirming the importance of epigenetic events in T cell fate determination.

DNA methylation, histone methylation and histone acetylation have been shown to play important roles in Th1/Th2 differentiation. Among these, extensive studies using chromatin immunoprecipitation (ChIP) demonstrated that Th1/Th2 differentiation is highly associated with changes in histone acetylation at different cytokine loci (55) (82-85). It has been shown that naïve $CD4^+$ T cells have almost no detectable histone acetylation on the *Ifng* and *Il4* loci. TCR signals initiate chromatin remodeling and locus opening in a cytokine-independent fashion. Subsequently, polarizing cytokine signals maintain and reinforce the accessible state at the relevant cytokine locus elicited by the TCR (55), (82). The polarized acetylation pattern of cytokine loci is maintained through more than 20 cell divisions, even in the absence of polarizing cytokine signals or TCR signals, and enhanced in response to restimulation (83). However, the polarized cytokine gene acetylation pattern in Th1/Th2 cells still has the capability to change if the polarized cells are cultured under opposite cytokine conditions (83). For example, after Th1 or Th2 clones are recloned with opposite polarizing conditions, the recloned cells display increased histone acetylaton at the formerly hypoacetylated cytokine loci with no reduction of histone acetylation at the originally hyperacetylated cytokine loci. These data indicate that hypoacetylation *per se* is not necessary to maintain irreversible gene silencing (83).

Transcription factors involved in epigenetic regulation of Th differentiation

During Th1 and Th2 cell differentiation, non-selective histone hyperacetylation is found at both the *Ifng* and *Il4* loci (55), (82). These early changes require TCR signaling but not polarizing cytokine signals. Therefore, Th1 and Th2 differentiation share a common initiation pathway emanated from TCR. TCR ligation leads to the release of intracellular calcium stores, stimulating the calcium-dependent phosphatase calcineurin that dephosphorylates NFAT. NFAT is a lineage non-specific transcription factor for Th1 and Th2 cell differentiation, which goes into the nucleus and non-selectively stimulates both IFN-γ and IL-4 production (86).

Lineage specific transcription factors are also involved in epigenetic regulation of Th differentiation, although the precise mechanisms through which these transcription factors mediate changes in chromatin are not clear. STAT proteins have been shown to be critical for chromatin remodeling of cytokine loci. In STAT4 deficient T cells, DNase hypersensitivity (23) and histone acetylation (82), (85) at the *Ifng* locus are significantly lower under Th1-skewing conditions. In the absence of STAT6, neither histone hyperacetylation (55) nor DNA demethylation (87) around the *Il4* locus can be fully established. STAT4 binds directly to the *Ifng* gene (38) and STAT6 to the *Il4* gene (55). How STAT4 and STAT6 mediate their functions remains unknown. Possible mechanisms include recruiting chromatin remodeling factors, acting as a cofactor of other transcription factors, or inducing the expression of master transcription factors, such as T-bet or GATA-3. The role of T-bet and GATA-3 in epigenetic changes has been actively studied. Overexpression of these factors results in substantial changes in chromatin

structure at the cytokine gene loci. Ectopic expression of T-bet induces DNase hypersensitivity of the *Ifng* gene and enhances *Ifng* gene transcription, even in the absence of STAT4 (23, 82). Likewise, overexpression of GATA-3 leads to alterations within the *Il4* gene, including the appearance of DNase hypersensitivity sites (69), histone acetylation(88) and DNA demethylation (1). Loss-of-function studies further confirmed the importance of these factors in epigenetic regulation. In T-bet knockout T cells, hyperacetylation of the *Ifng* promoter is impaired (55). In conditional GATA-3-deficient T cells, there is an increase in DNA methylation at the *Il4* locus and a decrease in histone hyperacetylation at the *Il5* locus (89). The order in which these transcription factors act and if this interaction is direct or not has not been elucidated.

Cis-regulatory elements in the *Ifng* gene

The *Ifng* gene is located on mouse chromosome 10 and human chromosome 12. IFN-γ expression is controlled at the transcription level. Regulation of transcription is a complex process that involves the binding of transcription factors to cis-regulatory elements that include the promoter and the more distal regulatory elements, such as enhancers, insulators and silencers. The proximal promoter is an important cis-regulatory module which specifies the site of transcription initiation. The distal regulatory elements are commonly located within around 50 kb upstream or downstream of the transcription initiation site of a gene, but they can be located up to several hundred kilobases away. In general, there are three methods to search for regulatory elements. The first method involves using bioinformatic approaches to find the conserved non-coding sequences

(CNS) between different species, since the important regulatory elements are usually conserved evolutionarily. The second method includes the utilization of a DNase I hypersensitivity assay to identify the relative open chromatin structures. These open chromatin structures are susceptible to digestion by DNase I and regulatory elements are commonly found in open chromatin to allow access of transcription factors to specific DNA sequences to control gene expression. The third method is to perform functional analysis, such as a promoter-reporter assay or the use of transgenic and knockout mice. Although bioinformatic approaches are useful for the initial identification of regulatory elements, a combination of bioinformatic and experimental analysis is required to establish whether a given CNS is actually utilized by a particular cell type.

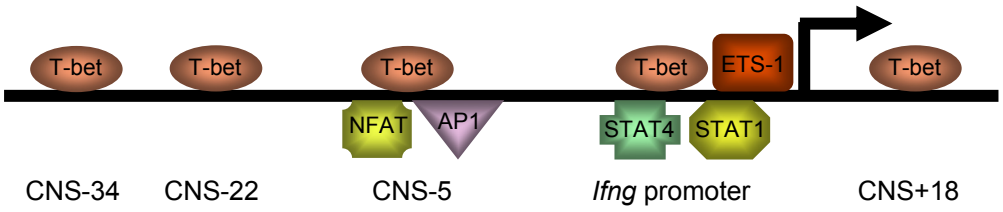
While the Th2 cytokine locus has been substantially studied and has emerged as a model to understand dynamic chromatin changes during Th2 cell differentiation, less is known about the *Ifng* locus. The promoter region of the *Ifng* gene is necessary for *Ifng* transcription. During transcription, DNase I hypersensitivity sites, histone acetylation and H3K4 dimethylation at the promoter region are significantly increased in a Th1 cell specific manner (81, 90), (85, 91). Th1 lineage specific transcription factors, T-bet and STAT4, have been demonstrated to bind to the proximal promoter of the *Ifng* gene and induce *Ifng* transcription (30), (31), (38) (Figure 1.6). However, studies of the proximal promoter and short upstream regions of the *Ifng* gene suggest that such elements are not sufficient for proper T cell specific expression. For instance, although it has been found that the immediate 108 bp 5' flanking sequence of the human *IFNG* promoter contains

two regulatory elements (92), and that these elements are necessary for activation-induced promoter function in both transient transfected Jurkat cells and primary human T cells, transgenes containing the *Ifng* promoter, *Ifng* introns and up to 3.4 kb of 5' flanking sequence of the *Ifng* gene, do not direct robust Th1 specific IFN-γ expression *in vivo* (93, 94). In contrast, transgenic studies using a 191 kb bacterial artificial chromosome containing 90 kb of 5' and 3' human *IFNG*-flanking sequence achieved high levels of Th1 specific human IFN-γ expression in murine T cells (94). These results suggest that distal transcription regulatory elements are dispersed in an extended region of over 100 kb surrounding the gene are required for the proper expression of *Ifng* transcripts.

Figure 1.6 Map of murine Ifng locus.

Conserved noncoding sequence (CNS) elements and their associated transcription factors at the *Ifng* locus are depicted.

Figure 1.6


T-bet
T-bet
T-bet
T-bet
ETS-1
T-bet
NFAT
AP1
STAT4
STAT1
CNS-34
CNS-22
CNS-5
Ifng promoter
CNS+18

Using large-scale cross-species DNA sequence comparison, several conserved noncoding regions near the *Ifng* gene have been identified. CNS-5 (or CNS1), which is located 5 kb upstream of the *Ifng* transcription initiation site, was discovered by two groups: Rao (95) and Wilson (90). This region has Th1-specific inducible DNase I hypersensitivity sites and significantly higher H3K4 methylation in Th1 cells compared with Th2 cells, which indicates a Th1-specific accessible chromatin structure at this region (95). T-bet and NFAT, the two transcription factors that are essential for Th1 differentiation, have been found to bind to CNS-5, suggesting that this region might regulate IFN-γ expression through interaction with these transcription factors (95) (Figure 1.6). Transient transfection assays show that this element has clear enhancer activity (90).

CNS+18 (CNS2) was identified by Wilson and colleagues (90). CNS+18 is located 18 kb downstream of the transcription initiation site of the *Ifng* gene. Like CNS-5, this region has strong enhancer activity for *Ifng* gene expression and acts synergistically with CNS-5. In Th1 cells, histone acetylation, H3K4 dimethylation and DNase I hypersensitivity sites are dramatically increased at this region, and these favorable transcriptional modifications are parallel to *Ifng* mRNA expression. T-bet is able to bind to this region, although the binding is weaker in comparison to CNS-5 (90) (Figure 1.6). A chromosome conformation capture (3C) assay shows that CNS-5 and CNS+18 interact with the proximal promoter region of the *Ifng* gene, presumably by looping (96).

CNS-22 is located 22 kb upstream of the *Ifng* transcriptional start site (91, 97). A promoter-reporter assay that coupled this element to the proximal *Ifng* promoter shows a

T-bet-dependent substantial increase in IFN-γ expression. A bacterial artificial chromosome transgenic reporter system demonstrates that conditional deletion of the CNS-22 element resulted in nearly complete loss of reporter expression in Th1 cells, cytotoxic T lymphocytes and NK cells. These data indicate that this single distal element may be essential for *Ifng* gene expression in both the adaptive and innate immune response. In contrast to other CNS elements, CNS-22 exhibits histone hyperacetylation in both Th1 and Th2 cells and contains a cluster of binding sites for transcription factors that are known to be important in Th1 and Th2 lineage determination, such as T-box factor, STAT, GATA, IRF, NF-κB and Ikaros. Among these transcription factors, T-bet has been demonstrated to bind to CNS-22 in both resting and stimulated Th1 cells (Figure 1.6). This suggests that CNS-22 may function as an activator or silencer in distinct lineages. In addition, naïve $CD4^+$ T cells display approximately twice the acetylated histones at the CNS-22 region in comparison to other sites examined, suggesting that this region may represent a chromatin entry site for transcription factors to carry out their functions (91, 98).

CNS-34 was identified by Weaver and colleagues (91) and exhibits increased acetylation in Th1 cells, and is bound by T-bet (91) (Figure 1.6). Although CNS-34 has T-bet-dependent *Ifng* enhancer activity *in vitro*, its *in vivo* function needs to be elucidated.

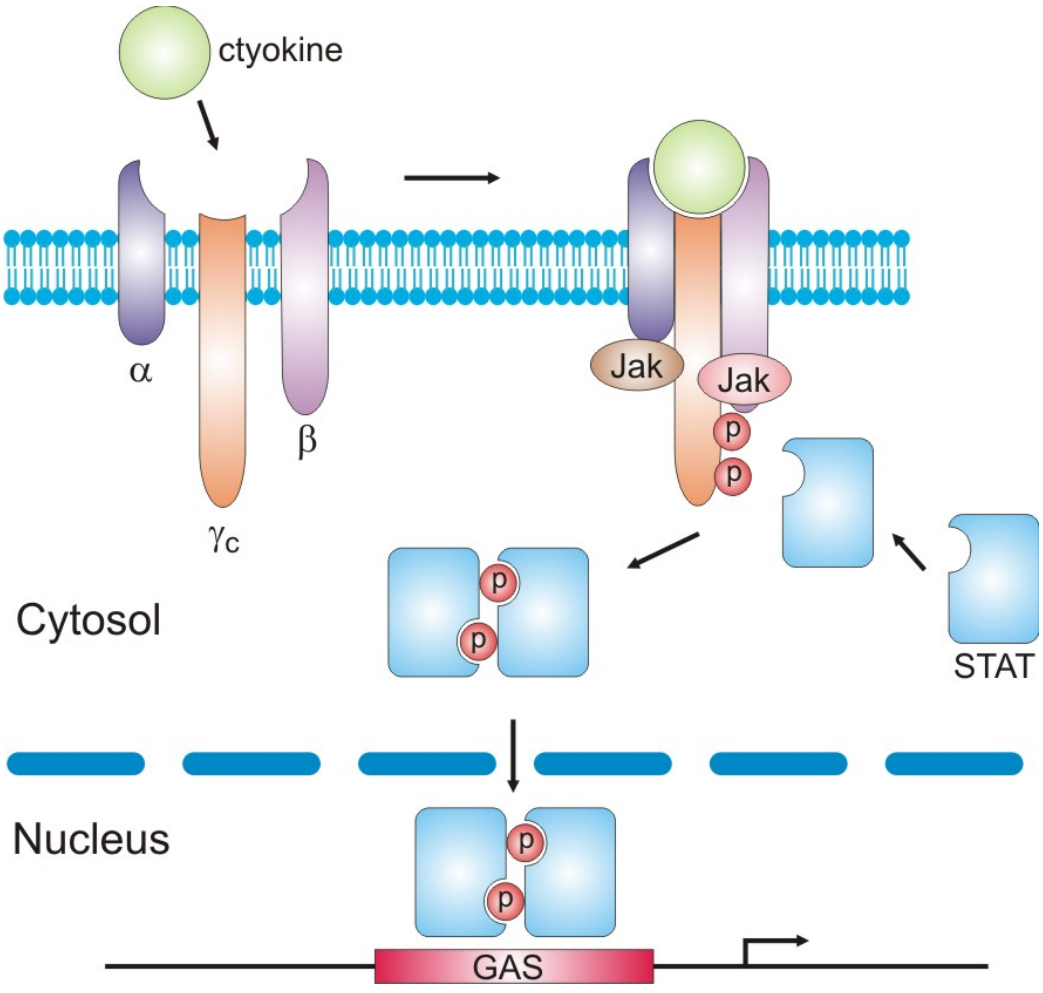
Jak-STAT pathway

As discussed above, cytokines are involved in the growth and differentiation of numerous cell types. They act by binding to specific receptors, which generally lack intrinsic tyrosine kinase activity and function through receptor-assocated kinases to mediate phosphorylaiton events. The Janus kinases are a family of receptor-associated tyrosine kinases that, together with STAT proteins, provide a rapid signaling pathway for cytokines. In general, cytokine receptor engagement recruits associated Jaks and results in Jak activation through auto-phosphorylation and trans-phosphorylation. Activated Jaks phosphorylate receptors to create docking sites for signaling proteins that have Src homology 2 (SH2) domains. Inactive STAT monomers bind to the phosphorylated receptors through their SH2 domain and are themselves phosphorylated by Jaks. Phosphorylated STATs dissociate from receptors, form dimers and subsequently translocate into the nucleus to induce gene transcription (Figure 1.7).

Figure 1.7 Jak-STAT pathway.

Binding of cytokines to their individual receptors activates specific Jaks, which in turn phosphorylate tyrosine-based docking sites on the receptors. STAT monomers bind to the docking site through SH2 domain, get phosphorylated, form dimers, and then translocate into the nucleus, where they bind and activate specific target genes.

Figure 1.7

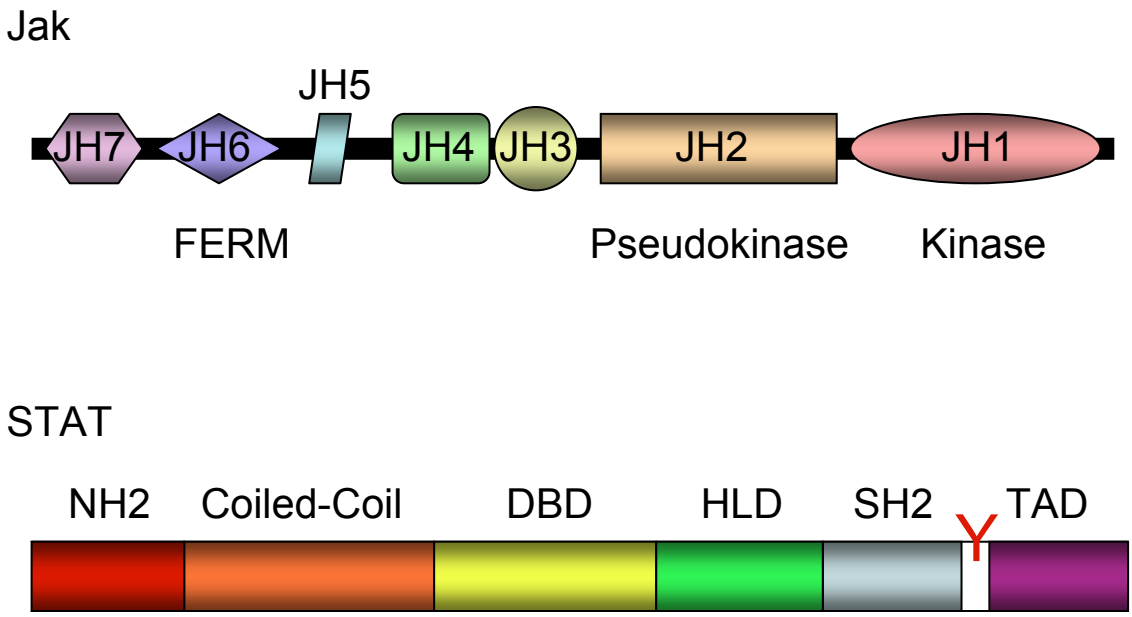


Four different Janus tyrosine kinases (Tyk2, Jak1, Jak2 and Jak3) have been identified and share significant structural homology with each other. Jaks have seven Janus homology (JH) domains (Figure 1.8). JH1 is a C-terminal kinase domain that is preceded by a pseudokinase domain, JH2. The psuedokinase domain has crucial regulatory activity without catalytic functions. The amino terminus of the Jaks comprises four JH domains (Half of JH4 and JH5-7) that are designed as FERM (four point one, ezrin, radixin, moesin) domain. The FERM domain mediates the association of Jaks with proline-rich regions on receptors. The first link between Jaks and cytokine signaling was made in 1992 when it was found that Tyk2 mediates signaling from the IFN α/β receptor to cytoplasmic transcription factors through analysis of Tyk2-mutant cell lines (99). Thereafter, it has been reported that Jaks are associated with a number of cytokine receptors and their special and essential functions are clarified by studying Jak knockout or conditional knockout mice (100).

Figure 1.8 Structure of Jak and STAT.

Regions of homology shared by Jaks have been termed Jak homology (JH) domains. JH1 is a kinase domain and JH2 is a pseudo-kinase domain. FERM domain consists half of JH4 and JH5-7. Stats have seven essential domains, including a transcriptional activation domain (TAD), a tyrosine activation domain (Y), an SH2 domain, a helical linker domain (HLD), a DNA binding domain (DBD), a coiled-coil domain and a NH2 domain.

Figure 1.8


Jak
JH5
JH7
JH6
JH4
JH3
JH2
JH1
FERM
Pseudokinase
Kinase
STAT
NH2
Coiled-Coil
DBD
HLD
SH2
Y
TAD

Seven STAT family members have been identified so far, each of which is composed of seven essential domains (Figure 1.8). These domains include a transcriptional activation domain (TAD) that is the most variable region and promotes gene transcription, a tyrosine activation domain (Y) that is phosphorylated by Jaks, an SH2 domain that binds to specific receptors, a helical linker domain (HLD) that ensures appropriate conformational changes upon the dimerization damain binding to DNA, a DNA binding domain (DBD) that is conserved among the STAT family members and directs binding to the γ interferon-activated site (GAS), a coiled-coil domain that binds the regulator proteins, and an NH2 domain that is involved in homotypic dimerization of inactive STATs, DNA binding, and the nuclear import or export process. STAT's specificity is largely determined by the binding preference of their SH2 domains to certain docking sites on cytokine receptors, although cell type and differentiation state also contribute. In addition, forming heterodimers, tetramers and other higher order complexes expand the range of STAT/DNA binding specificities (101)

The Jak-STAT pathway provides a rapid signal that is tightly controlled by various regulatory systems to avoid excessive simulation. These regulatory molecules include protein tyrosine phosphatases (102-104), suppressors of cytokine signaling (SOCS) (105) and protein inhibitors of activated STATs (PIAS) (106). In addition, endosomal degradation of Jak/receptor complexes through receptor-mediated endocytosis (107) (108), ubiquitination (109) and nuclear export also play a role in regulating Jak-STAT signal strength (110), (111).

Jak3

In contrast to other Jaks that are ubiquitously expressed in tissues, Jak3 is preferentially expressed in hematopoietic cells, where it associates specifically with the common gamma chain (γc) that is shared by the receptors for γc cytokines, such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines are crucial for the development, homeostasis, effector function and memory generation of immune cells.

The function of Jak3 has been illustrated by analysis of Jak3 knockout mice generated by several groups (112-114). These mice have severe combined immunodeficiency (SCID) that resembles the phenotype of γc mutant mice. Jak3 knockout mice have very small thymuses, absence of lymph nodes, no NK cells or γ/δ T cells and reduced number of α/β progenitor cells. Analysis of the peripheral cells in Jak3-deficient mice shows that there are few $CD8^+$ T cells and normal number of $CD4^+$ T cells. However, the majority of $CD4^+$ T cells have activated or memory phenotype and are $CD4^+$ $CD44^{high}$ $CD62L^{low}$. In contrast to SCID patients, the number of B cells in Jak3 knockout mice is also profoundly decreased (112-114).

The phenotype observed in Jak3-deficient mice can be readily interpreted by the ever-growing knowledge of cytokine biology through inactivating γc cytokines or γc cytokine receptors. The absence of NK cells in Jak3-deficient mice can be explained by the failure of IL-15 signaling. IL-15 knockout mice lack NK cells, suggesting IL-15 is critical for

the development and survival of these cells (115), (116, 117). The lymphocyte defects in Jak3 knockout mice are mostly explained by the deficiency of IL-7 signaling. IL-7 is critical for early lymphocyte development through two signals. One signal is for the proliferation and survival of lymphocyte precursors by inducing the expression of anti-apoptosis factor Bcl2 and activating phosphatidylinositol (PI3) kinase (118, 119). The other signal is for generation of lymphocyte precursors by promoting V(D)J recombination of TCR and Immunoglobulin genes (120-122). Therefore, in mice lacking IL-7, IL-7R or Jak3 mice, γ/δ T cells fail to develop, B cell development is blocked at pro-B stage and α/β T cell progenitors are nearly absent. In addition to lymphopoiesis, IL-15 and IL-7 are important for the homeostatic proliferation of naïve and memory $CD8^+$ T cells (123, 124) and naïve $CD4^+$ T cells (125). However, the proliferation of memory $CD4^+$ T cells is less dependent on both cytokines (126). This may explain the phenotype of fewer $CD8^+$ T cells but more memory-like $CD4^+$ T cells in the periphery of Jak3 knockout mice.

Although phenotypically the majority of peripheral T cells in Jak3 knockout mice are activated- or memory-like cells, their function is unique. First, they fail to proliferate and have impaired survival in response to stimulation *in vitro* (112, 127). Second, they produce little IL-2 or IL-4, but produce IL-10, IFN-γ and TGF-β instead when stimulated *in vitro* (128). Third, they modestly suppress the proliferation of wild type $CD4^+$ T cells in coculture assays (128). Taken together, $CD4^+$ T cells in Jak3 knockout mice display

the features of regulatory T cells, indicating that Jak3 is not critical for directing $CD4^+$ T cell differentiation into a regulatory lineage.

As detailed above, $CD4^+$ T cells in mice lacking Jak3 have undergone an alternative pathway of differentiation. To investigate the role of the Jak3/γc pathway in naïve $CD4^+$ T cell activation, homeostatic proliferation and effector function, Di Santo and colleagues crossed γc-deficient mice to TCR transgenic mice to generate naïve $CD4^+$ T cells. They found that in contrast to memory T cells, naïve $CD4^+$ T cells cannot survive in the absence of γc signaling (125). This survival defect is able to be rescued by overexpression of human BCL2 (129). The proliferation of naïve $CD4^+$ T cells is independent of γc signals both *in vivo* (125) and *in vitro* (129, 130), which challenged the old dogma that IL-2 and IL-4 are T cell growth factors. This group also demonstrated that γc cytokines are involved in the full differentiation of $CD4^+$ T cells *in vivo* by providing the evidence that γc-deficient $CD4^+$ T cells fail to reject the skin graft and have defects in producing IFN-γ or granzyme B (130). The mechanisms remain unclear.

STAT5 and the mechanisms of its transcriptional activation

The predominant STAT that is activated by γc cytokines is STAT5. Encoded by two distinct but closely related genes, the two isoforms of this transcription factor, STAT5a and STAT5b, are 91% identical at the amino acid level with the extreme carboxyl-terminal transactivation domain showing the most divergence. However, their physiological functions are not fully redundant. For example, STAT5a knockout mice

display a profound defect in adult mammary gland development and lactogenesis (131), whereas STAT5b-deficient mice exhibit defects related to growth hormone actions (132). Both STAT5a and STAT5b knockout T cells show decreased proliferation in response to stimulation, although the defect in the cells lacking STAT5b is more severe (133, 134). Both STAT5a and STAT5b contribute to normal NK cell development with a greater defect in NK cell function seen in STAT5b knockout mice (134). In general, with respect to immune system, deficiency of STAT5a or STAT5b alone does not develop severe defects. STAT5ab double knockout mice have severely impaired lymphocyte development, survival and function, resembling the phenotype of Jak3 and γc knockout mice (135). These suggest that these two closely related isoforms regulate the expression of some distinct target genes as well as the identical ones.

The molecular mechanisms by which STAT5 promotes gene transcription have been studied. Initially, STAT5 was shown to have transactivation activity by binding to GAS and associating with several cofactors such as p300/CBP, N-myc interactor (Nmi), protein inhibitors of activated STAT (PIAS), and SMRT. P300/CBP and Nmi have been reported to potentiate STAT5 mediated transactivation, whereas PIAS and SMRT down-modulate expression of STAT5 target genes (136, 137). Therefore, STAT5-interacting proteins are able to function as positive or negative factors to regulate STAT5 signaling and contribute to the specificity and complexity of STAT5-mediated gene activation.

Some STAT5 cofactors have histone modification activity, which implicates STAT5 in chromatin regulation during cytokine-induced transcription. For instance, it is known that p300/CBP has intrinsic histone acetyltransferase activity (138, 139), and SMRT recruits HDACs to cause transcriptional repression (140, 141). Recently, STAT5 itself has also been shown to have chromatin remodeling activity. Studies in B cells and γδ T cells have shown that STAT5 directly regulates chromatin remodeling by promoting histone acetylation of genes including CIS (142), the distal V_HJ558 gene (122) and the TCRγ locus (143). In addition, STAT5 interacts with SWI/SNF chromatin remodeling factors on the β- and γ-casein promoters in mammary epithelial cells following prolactin plus extracellular matrix protein stimulation (144). Whether the chromatin remodeling activity of STAT5 is the direct result of STAT5/DNA binding or the recruitment of a chromatin remodeling complex by STAT5 remains to be elucidated.

Work presented in this thesis

The goal of my thesis is to further understand the importance of γc cytokines in T cell immunity. Specifically, we investigated the role of γc cytokines in $CD4^+$ T lymphocyte proliferation, survival and differentiation. Using naïve T cells lacking Jak3-dependent cytokine signals, we demonstrate that although Jak3-dependent cytokine signals are dispensable for $CD4^+$ T cell proliferation, they are indispensable for $CD4^+$ T cell differentiation and survival. This work elucidates the differential mechanisms regulating clonal expansion, survival and effector function of T cells. Furthermore, this work

identified a novel mechanism by which Jak3-STAT5 pathway regulates Th1 differentiation.

CHAPTER II

Janus-Kinase-3-Dependent Signals are not Required for the Cell Cycle Progression of Naïve $CD4^+$ T Cells

Introduction

T cell proliferation is essential for mounting an effective adaptive immune response. A key element of proliferation is the entry of cells into the cell cycle, a complex process that is tightly controlled by the ordered expression of cyclins, the activation of cyclin-dependent kinase (Cdk) enzymatic activity and the subsequent phosphorylation of relevant substrates. The first cyclin expressed during the G1 phase is a D-type cyclin, which is a rate-limiting factor for cell cycle progression from the G1 to the S phase. The induction of cyclin E occurs at the late G1 restriction point, and cyclin A is expressed at S phase entry (145). The activity of Cdks is stimulated by cyclins and inhibited by cyclin-dependent kinase inhibitors (CDKI), such as p27kip1. Cyclin/Cdk complexes phosphorylate the retinoblastoma (Rb) gene product, leading to the activation of the E2F transcription factor, which is required for the transcription of S phase genes.

T cell proliferation is induced following stimulation of the T cell receptor (TCR) and costimulatory molecules; in addition, cytokines such as IL-2 and IL-4, that signal through receptors sharing the common γ (γc) chain, have been shown to promote lymphocyte proliferation (146). Among these, IL-2 has long been recognized as the most potent T cell growth factor (147). *In vitro* studies have shown that IL-2 very efficiently promotes the growth of antigen-activated T cells (148, 149). Antigen- or mitogen-induced T cell proliferation *in vitro* can be substantially inhibited using monoclonal antibodies specific for IL-2 or the IL2R, suggesting that IL-2 is an essential element in T cell proliferation (150-152). In later studies, it was found that IL-2 promotes the transit of T cells through

G1 to S phase of the cell cycle by up-regulating cyclin D2, cyclin D3, cyclin E and E2F, and down-regulating p27kip1 (153-156). Based on these findings, among others, the consensus view is that TCR and CD28 stimulation induce quiescent T cells to leave G0 and enter the G1 phase of the cell cycle (157); in addition, these signals induce the expression of the high-affinity IL-2 receptor and stabilize the IL-2 message, rendering the cells competent for IL-2-driven proliferation.

Recent studies performed in intact animals have challenged this view and demonstrated IL-2- or γc cytokine-independent T cell expansion *in vivo*. When adoptively-transferred, IL-2-deficient or IL-2R-deficient DO11.10 T cells challenged with OVA peptide underwent comparable expansion compared to wild type T cells (158, 159). Similarly, after correcting the autoimmune defect in IL-2Rβ-deficient mice by selective expression of IL-2Rβ in the thymus, IL-2R $\beta^{-/-}$ T cells also showed normal expansion during both primary and secondary immune responses (30). Finally, Di Santo and colleagues reported that naïve γc chain-deficient T cells proliferate robustly in response to antigenic stimulation in *vivo* (125). Together, these results indicate that γc cytokine signals are not absolutely required *in vivo* for T cell proliferation.

Several *in vitro* studies also suggest that T cell proliferation can occur in an IL-2-independent manner. For instance, except under conditions of suboptimal stimulation, IL-2 or IL-2R antibody blockade cannot completely inhibit T cell proliferation (73, 160). Further, IL-2- or IL-2R-deficient T cells can be induced to proliferate in response to

specific antigens or mitogens, although the proliferation is generally reduced compared with that of control T cells (161-164). Finally, several studies have suggested that TCR plus CD28 stimulation controls cell cycle progression independently of IL-2. Using IL-2 or IL-2R blocking antibodies, or IL-2-deficient cells, these studies indicated that TCR/CD28 engagement could promote T cell proliferation by inducing the expression of cyclin D and cyclin E, enhancing the transcriptional activity of E2F, and down-regulating the inhibitory function of p27kip1 (165-171).

However, there are a number of caveats with these studies that have hampered the general acceptance of the view that T cell proliferation does not require IL-2 or other γc cytokine signals. First, the failure to completely block T cell proliferation with IL-2 or IL-2R antibodies may reflect the lower affinity of these interactions relative to the affinity of IL-2 for its receptor. Second, several of these studies were performed using tumor cell lines or anergic T cells, which may not reflect the requirements of primary naive T cells. A similar concern applies to the studies using IL-2- or IL-2R-deficient T cells, as these cells are also not naïve T cells (158). Finally, these *in vitro* studies did not rule out the possibility that T cell proliferation was being induced by γc cytokines other than IL-2; although IL-2 is the main γc cytokine that is secreted when T cells are initially activated *in vitro*, IL-4 and IL-21 are also produced by activated T cells and can promote T cell proliferation (172-174). Therefore, none of these studies conclusively demonstrated that naïve T cell proliferation was γc cytokine-independent.

In this report, we investigated the requirement of γc cytokines in the proliferation and cell cycle control of primary naïve T cells *in vitro*. We analyzed the proliferation of naïve $CD4^+$ T cells from the mice lacking Janus kinase 3 (Jak3), a tyrosine kinase that is essential for signaling via all γc cytokine receptors. We complemented these experiments with analysis of wild type naïve $CD4^+$ T cells treated with a pharmacological inhibitor of Jak3. Together, these studies demonstrated that Jak3-dependent γc cytokine signals are not required for naïve primary $CD4^+$ T cell proliferation and cell cycle regulation *in vitro*.

Results

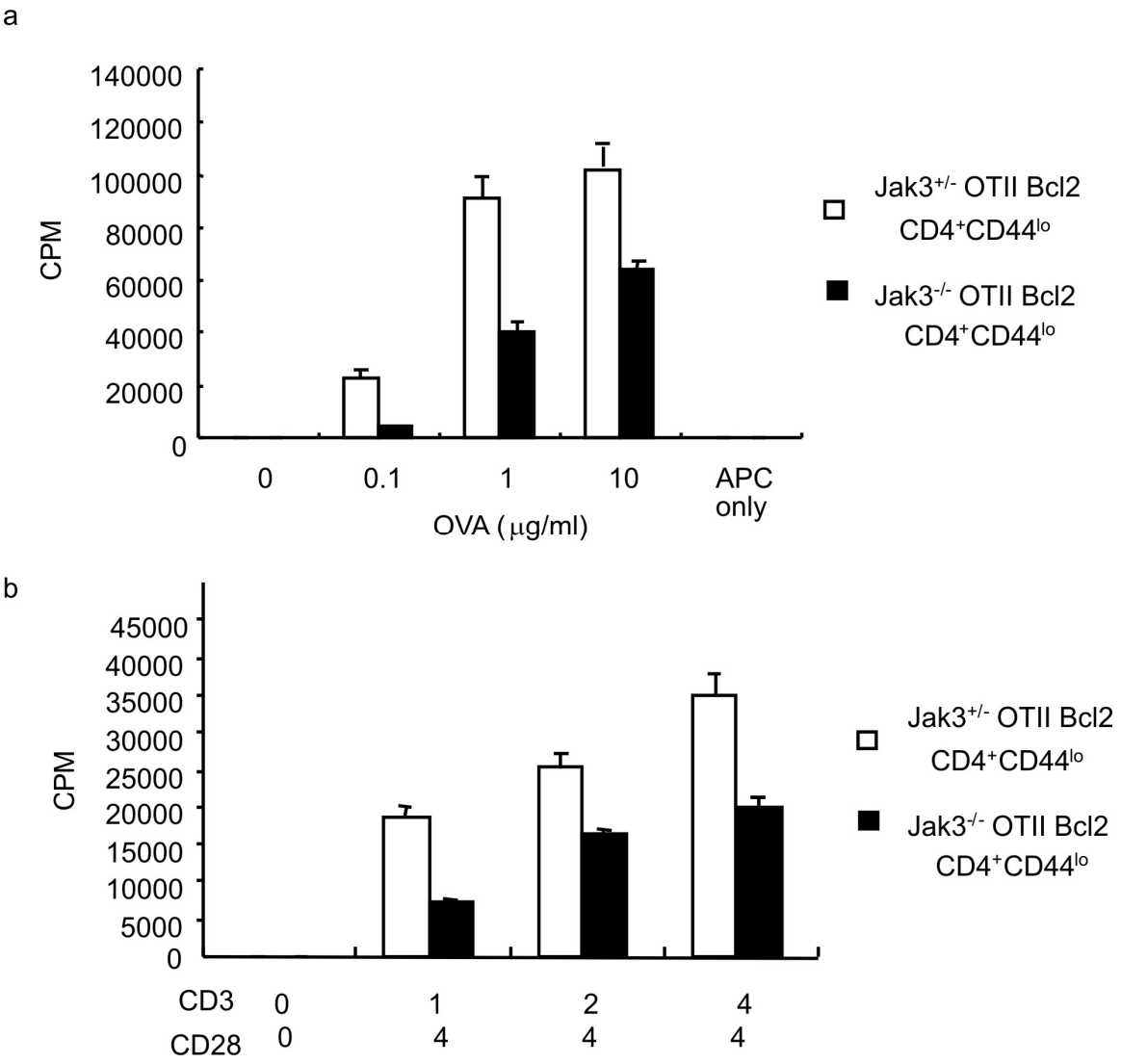
Jak3-dependent cytokine signals are required for optimal T cell responses *in vitro*.

As mentioned in the introduction part of this thesis, the majority of $CD4^+$ T cells in *Jak3*$^{-/-}$ mice have an activated or memory phenotype. To investigate the potential role of Jak3-dependent cytokine signals in naïve T cell proliferation, we established an *in vitro* assay system using homogenous populations of naïve *Jak3*$^{-/-}$ $CD4^+$ T cells. Previous work has generated naïve $CD4^+$ peripheral T cells by crossing *Jak3*$^{-/-}$ mice to TCR transgenic mice (125). Here, we crossed *Jak3*$^{-/-}$ mice to the transgenic line expressing the OT-II TCR to obtain *Jak3*$^{-/-}$ $CD4^+$ T cells with a receptor specific for OVA peptide presented by the MHC class II molecule I-A^b. The majority of peripheral $CD4^+$ T cells in these mice have a naïve phenotype. However, the absolute number of T cells is greatly reduced. Since γc cytokines are required for naïve $CD4^+$ T cell survival *in vivo* mainly through the function of Bcl-2 (125), we also introduced a transgene expressing Bcl-2. The resulting *Jak3*$^{-/-}$ OT-II Bcl-2 mice have a large number of peripheral $CD4^+$ T cells which are $CD44^{lo}$. Purified $CD4^+$ $CD44^{lo}$ T cells from *Jak3*$^{+/-}$ or *Jak3*$^{-/-}$ OT-II Bcl-2 mice were stimulated with varying concentrations of OVA peptide presented by mitomycin C-treated antigen presenting cells (APCs) from C57BL/6 mice (Figure 2.1a) or anti-CD3 and anti-CD28 antibodies (Figure 2.1b). As shown, *Jak3*$^{-/-}$ $CD4^+$ $CD44^{lo}$ T cells were capable of proliferating in response to all stimuli, although the magnitude of the response was reduced in comparison to that of control cells. This reduced proliferation of *Jak3*$^{-/-}$ T cells could result from impaired T cell proliferation or impaired T cell survival, or both.

Figure 2.1 CD4+ T cell proliferation appears reduced in the absence of Jak3-dependent cytokine signals.

$CD4^{+}CD44^{lo}$ T cells were sorted from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice and stimulated with either mitomycin C-treated C57BL/6 APCs plus the indicated concentrations of $OVA_{323-339}$ (a) or various concentrations of anti-CD3 and anti-CD28 antibodies (b) for 48h, then pulsed with [3H]-thymidine for the final 18h. Cell proliferation was measured by [3H]-thymidine incorporation. Data represent the mean ± SE of the triplicate reactions. Statistically significant differences were seen between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ cells. The significant level is $P < 0.05$.

Figure 2.1


a
140000
120000
100000
80000
60000
40000
20000
0
CPM
0
0.1
1
10
APC only
OVA (μg/ml)
Jak3+/- OTII Bcl2 CD4+CD44lo
Jak3-/- OTII Bcl2 CD4+CD44lo
b
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
CPM
CD3 0 1 2 4
CD28 0 4 4 4
Jak3+/- OTII Bcl2 CD4+CD44lo
Jak3-/- OTII Bcl2 CD4+CD44lo

T cell proliferation is not affected by Jak3-dependent cytokines, but survival is

γc cytokines, especially IL-2, IL-4 and IL-7, promote T cell survival by up-regulating the anti-apoptotic factor Bcl-2 (175-178). In Jak3- or γc-deficient T cells, the expression of Bcl-2 is greatly decreased (125), (70). To determine whether constitutive expression of Bcl-2 is sufficient to reverse the survival defect of *Jak3*$^{-/-}$ T cells *in vitro*, we examined T cells for evidence of apoptosis. For these studies we used $CD4^{+}CD8^{-}$ single-positive (CD4SP) thymocytes from *Jak3*$^{+/-}$ or *Jak3*$^{-/-}$ OT-II Bcl-2 mice as a source of homogeneous naïve T cells. Following 3 days of stimulation, cells from *Jak3*$^{-/-}$ mice showed a significantly higher degree of cell death compared to control cells (Figure 2.2a). These findings indicate that Jak3-dependent cytokine signals normally induce a survival pathway that cannot be compensated for by constitutive expression of Bcl-2. Thus, in the absence of Jak3, the reduced level of ^{3}H-thymidine incorporation following T cell activation may be due to enhanced apoptosis, rather than impaired proliferation.

T cell proliferation can be assessed using the fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), providing a means of excluding dead cells in the population and of visualizing the proportion of cells representing each successive cell division cycle. As can be seen, after 3 days of stimulation with anti-CD3 and anti-CD28 antibodies, there was no significant difference in the percentage of divided cells between Jak3-deficient and Jak3-positive T cells, suggesting that Jak3-dependent cytokine signals do not regulate the proportion of cells entering the cell cycle (Figure 2.2b, c). To examine this issue more carefully, we determined the percentage of cells in each round of cell division, and

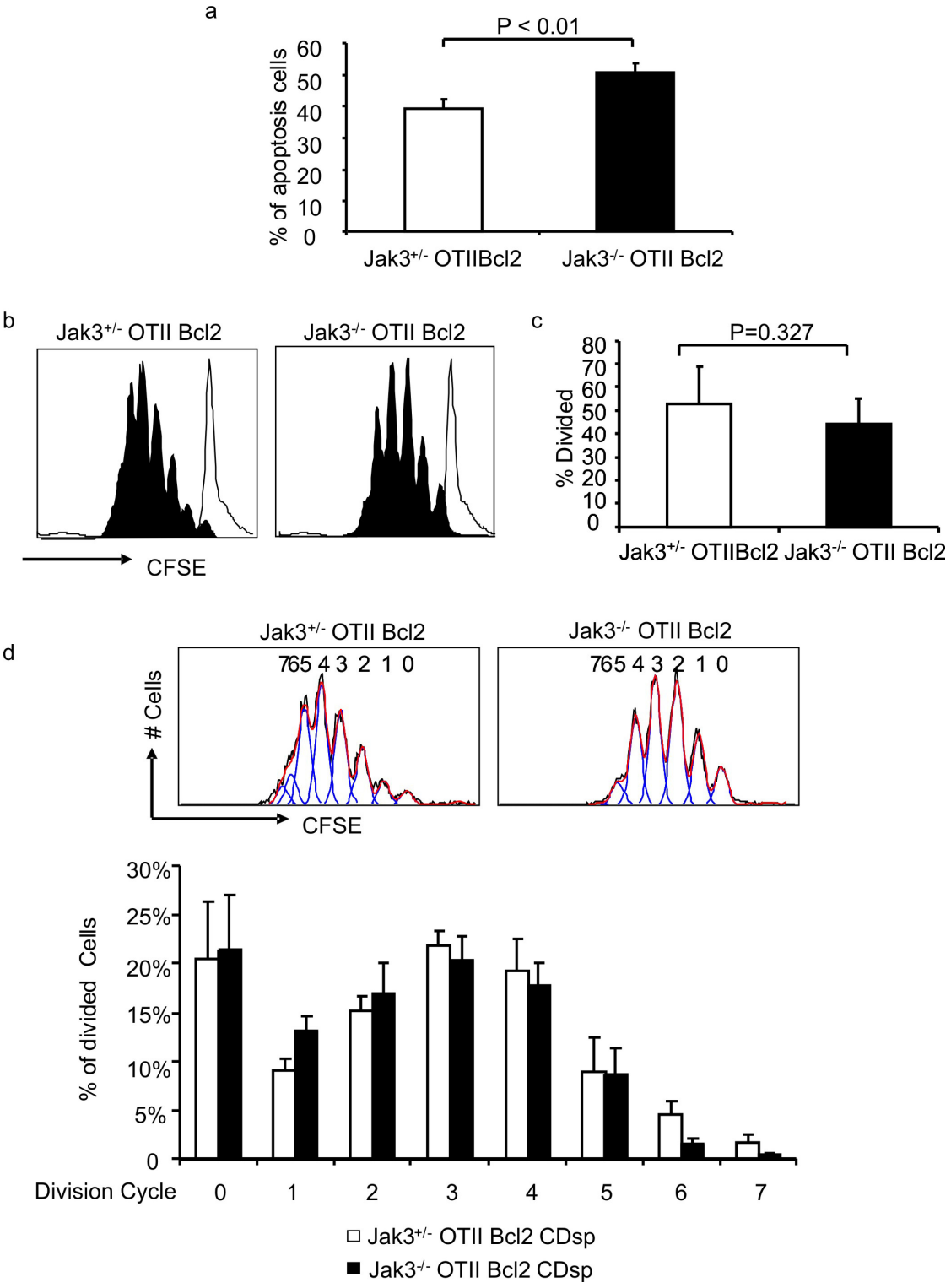
compiled the data from four independent experiments (Figure 2.2d). This analysis confirmed that T cells lacking Jak3 showed comparable proliferative capacity to T cells expressing Jak3. Similarly, a comparison of the proliferation index as well as the division index between the two populations of T cells indicated no significant differences in the average number of divisions that cells of each genotype underwent. Taken together, these data indicate that Jak3-dependent cytokine signals are not required for naïve T cell proliferation *in vitro*, but are important in maintaining maximum T cell survival.

Figure 2.2 Jak3-dependent cytokine signals affect T cell survival but not proliferation.

(a) Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) antibodies. Three days later, cells were stained with AnnexinV and 7-AAD, and analyzed by flow cytometry. Apoptotic cells were identified as AnnexinV+ 7-AAD+.

(b, c, d) CD4SP thymocytes were isolated from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice, labeled with CFSE, stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) antibodies, and analyzed by flow cytometry. b) representative histograms show the degree of CFSE dilution in *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 T cells. c) Percentage of *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 T cells that underwent cell division; data represent the mean ± SE from four independent experiments. d) Histograms were analyzed using the proliferation platform of the FlowJo software to estimate the percentage of cells in each round of cell division; representative data are shown above. The graph depicts the mean ± SE using data from four independent experiments. No significant differences were seen between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ cells.

Figure 2.2


a
P < 0.01
% of apoptosis cells
60
50
40
30
20
10
0
Jak3+/- OTIIBcl2
Jak3-/- OTII Bcl2
b
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
CFSE
c
P=0.327
% Divided
80
70
60
50
40
30
20
10
0
Jak3+/- OTIIBcl2
Jak3-/- OTII Bcl2
d
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
7 6 5 4 3 2 1 0
Cells
CFSE
% of divided Cells
30%
25%
20%
15%
10%
5%
0
Division Cycle
0
1
2
3
4
5
6
7
Jak3+/- OTII Bcl2 CDsp
Jak3-/- OTII Bcl2 CDsp

Pharmacological inhibition of Jak3 impairs T cell survival, but not proliferation

To rule out the possibility that *Jak3*$^{-/-}$ T cells are developmentally abnormal, leading to their independence from Jak3-dependent cytokine signals for T cell proliferation, we examined the responses of *Jak3*$^{+/+}$ CD4^{+} T cells treated with a small molecule inhibitor of Jak3, PS078507 (179). Compound PS078507 was developed by Pharmacopeia, Inc. (Princeton, NJ), and had an IC50 of 2.1nM for inhibition of Jak3 enzyme activity (Figure 2.3a). Inhibitory activity (IC50) on the other Jak kinases was measured at 20nM, 6.3nM, and 12nM for Jak2, Jak1, and Tyk2, respectively, and was >300nM on a panel of 30 additional kinases tested (data not shown). In a cellular assay examining inhibition of IL-2-induced proliferation of human peripheral blood T cells, the IC50 is 73nM (Figure 2.3b). The optimal concentration of PS078507 for Jak3 inhibition in murine peripheral CD4^{+} T cells was determined by examining IL-2-induced STAT5 phosphorylation (Figure 2.3c).

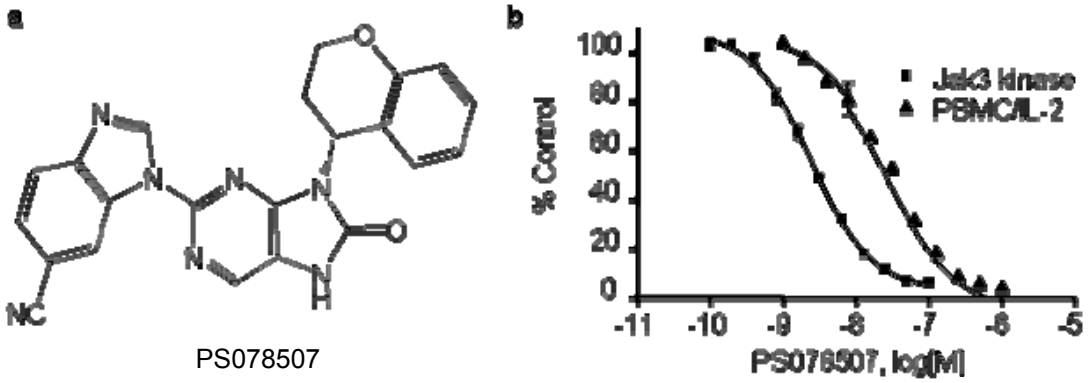
Figure 2.3 Characterization of the Jak3 inhibitor, PS078507

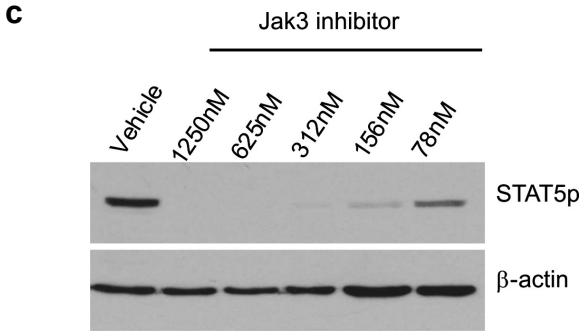
(a) Structure of the Jak3 inhibitor, PS078507.

(b) Various concentrations of PS078507 were included in the JAK3 kinase and cell proliferation assays as described below. Each compound dilution was tested in duplicate. The average IC50 values ± S.D for inhibition of human JAK3 kinase activity and IL-2-induced PBMC proliferation by PS078507 are 2.1±0.4 nM (n=3) and 73±30 nM (n=5), respectively.

(c) $CD4^+$ T cells were isolated from *Jak3*$^{+/+}$ mice, stimulated with anti-CD3 and anti-CD28 antibodies for 2 days, rested for 4 hours, incubated with vehicle alone or serial-diluted PS078507 for 30 minutes, then stimulated with IL-2 (50 ng/ml) for 15 minutes. Cell lysates were prepared and immunoblotted for pSTAT5 and β-actin.

Figure 2.3


a
b
PS078507
% Control
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
PS078507, log[M]
Jak3 kinase
PBMC/IL-2


c
Jak3 inhibitor
Vehicle
1250nM
625nM
312nM
156nM
78nM
STAT5p
β-actin

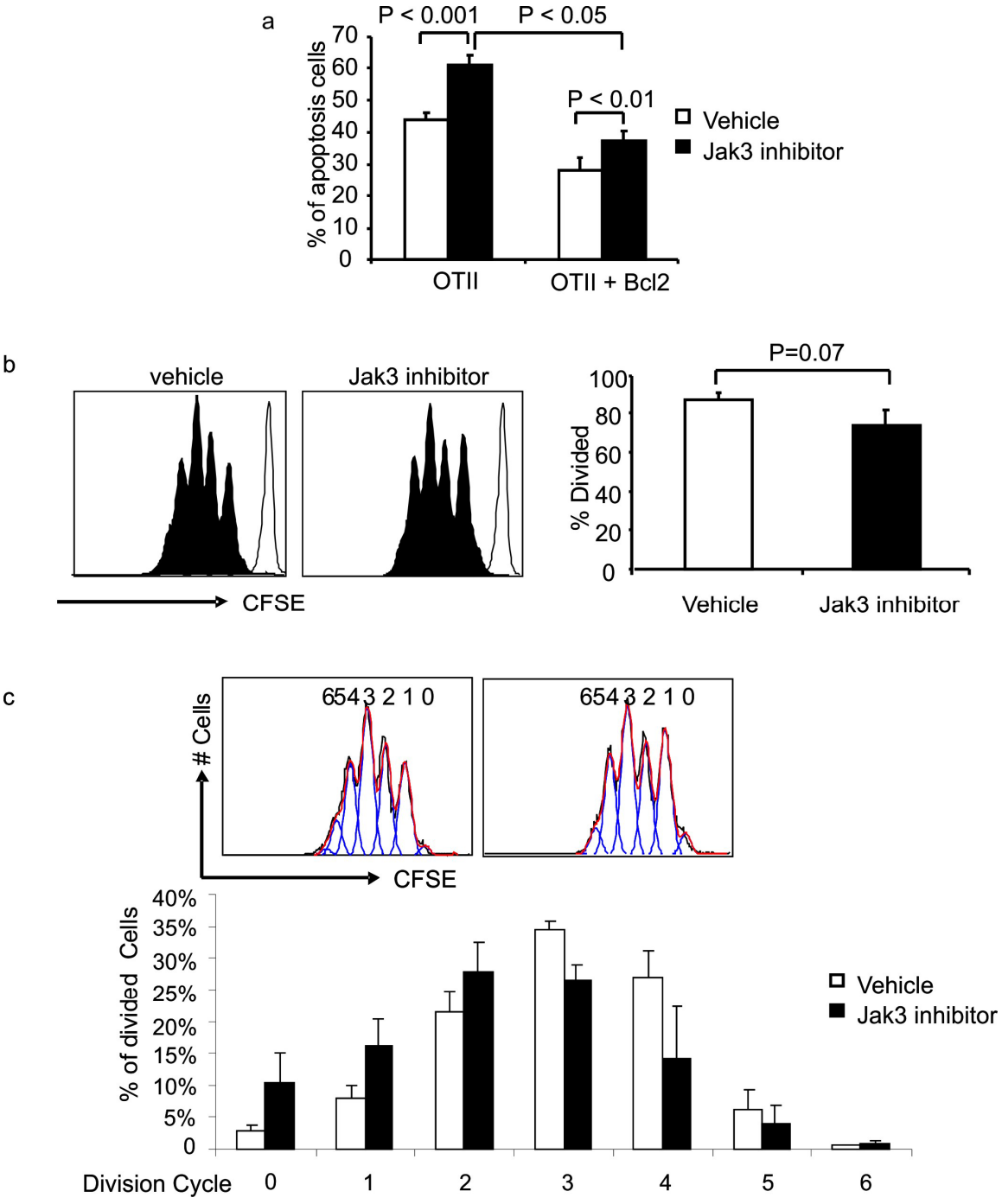
The effect of PS078507 on cell survival was then assessed following 3 days of *in vitro* stimulation. As shown, naïve *Jak3*$^{+/+}$ CD4^{+} T cells from OT-II transgenic mice displayed dramatically higher levels of apoptosis in the presence of the inhibitor, regardless of the expression of Bcl-2 (Figure 2.4a). Consistent with the data shown above using *Jak3*$^{-/-}$ T cells, when cell proliferation was determined using CFSE dilution, *Jak3*$^{+/+}$ naïve CD4^{+} T cells treated with PS078507 showed a comparable proportion of divided cells compared to cells treated with vehicle alone (Figure 2.4b). Further, the percentage of divided cells in each successive cell division cycle also showed no significant difference compared to controls, when averaged over four independent experiments (Figure 2.4c). Together, these data indicate that cell survival, but not proliferation, is regulated by Jak3-dependent cytokine signals.

Figure 2.4 CD4+ T cell survival but not proliferation is reduced by pharmacological inhibition of Jak3 activity.

(a) Purified $CD4^+$ splenocytes from OT-II-transgenic or OT-II Bcl-2-transgenic mice were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (4 μg/ml) antibodies for 3 days in the presence of vehicle alone or PS078507 at 312 nM, stained with AnnexinV and 7-AAD, and analyzed by flow cytometry. Apoptotic cells were identified as AnnexinV+ 7-AAD+.

(b, c) Purified $CD4^+$ splenocytes from OT-II-transgenic mice were labeled with CFSE and stimulated with anti-CD3 and anti-CD28 antibodies for 3 days in the presence of vehicle alone or PS078507 at 312 nM, and then analyzed by flow cytometry. c) Histograms show CFSE fluorescence. The bar graph indicates the percentage of T cells undergoing division in the absence or presence of PS078507; data show the mean ± SE compiled from four independent experiments. d) Histograms were analyzed using the proliferation platform of the FlowJo software to estimate the percentage of cells in each round of cell division; representative data are shown above. The graph depicts the mean ± SE using data from four independent experiments. No significant differences were seen between cells stimulated in the presence or absence of the Jak3 inhibitor.

Figure 2.4


a
P < 0.001
P < 0.05
P < 0.01
% of apoptosis cells
70
60
50
40
30
20
10
0
OTII
OTII + Bcl2
Vehicle
Jak3 inhibitor
b
vehicle
Jak3 inhibitor
CFSE
P=0.07
100
80
60
40
20
0
% Divided
Vehicle
Jak3 inhibitor
c
Cells
6 5 4 3 2 1 0
6 5 4 3 2 1 0
CFSE
40%
35%
30%
25%
20%
15%
10%
5%
0
% of divided Cells
Division Cycle
0
1
2
3
4
5
6
Vehicle
Jak3 inhibitor

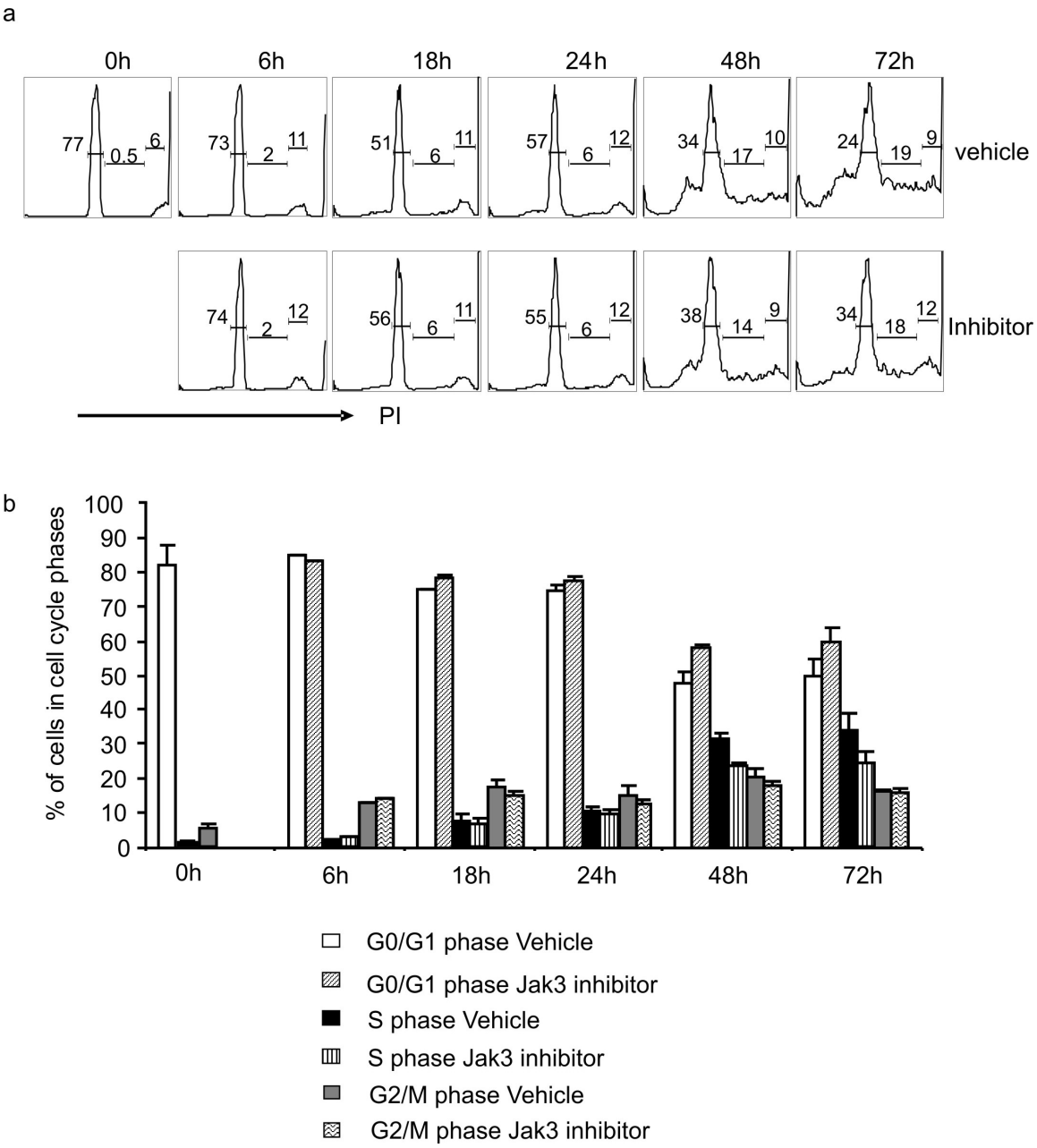
Cell cycle regulation is independent of Jak3 signaling

The precise function of TCR/CD28 signaling versus cytokine signaling in regulating naïve $CD4^+$ T cell proliferation *in vitro* remains ambiguous. To address this issue, we examined cell cycle progression of naïve T cells stimulated in the presence or absence of the Jak3 inhibitor, PS078507. $Jak3^{+/+}$ CD4+ T cells from OT-II transgenic mice were stimulated, harvested at a variety of timepoints, and analyzed for DNA content using propidium iodide (PI). The intensity of the PI signal is directly proportional to DNA content, with the rightmost peak on the histogram representing the cells in the G2/M phase, the leftmost peak showing the cells in the G0/G1 phase, and the area between the two peaks indicating the cells within the S phase (Figure 2.5a). To compare cell cycle progression between samples, we calculated the percentage of live cells in each phase, and averaged the data from three independent experiments (Figure 2.5b). This analysis indicated that CD4+ T cells lacking Jak3-dependent cytokine signals have indistinguishable cell cycle kinetics relative to cells receiving these cytokine signals ($P > 0.05$).

Figure 2.5 Cell cycle progression is not affect by Jak3 inhibition.

Purified $CD4^+$ splenocytes from OT-II-transgenic mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of vehicle alone or PS078507 at 312 nM. At the indicated time points, cells were harvested, fixed, stained with propidium iodide (PI) and analyzed by flow cytometry. *A,* Histograms represent the DNA content of the cells after stimulation in the presence of vehicle or PS078507. *B*, Percentage of cells in G0/G1, S and G2/M phase is indicated for each timepoint; data represent the mean ± SE compiled from three independent experiments. No significant differences were seen between cells stimulated in the absence versus the presence of the Jak3 inhibitor.

Figure 2.5


a
0h
6h
18h
24h
48h
72h
77
0.5
6
73
2
11
51
6
11
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12
34
17
10
24
19
9
vehicle
74
2
12
56
6
11
55
6
12
38
14
9
34
18
12
Inhibitor
PI
b
% of cells in cell cycle phases
100
90
80
70
60
50
40
30
20
10
0
0h
6h
18h
24h
48h
72h
G0/G1 phase Vehicle
G0/G1 phase Jak3 inhibitor
S phase Vehicle
S phase Jak3 inhibitor
G2/M phase Vehicle
G2/M phase Jak3 inhibitor

Cyclins and cyclin-dependent kinase inhibitors (CDKI) are the key regulators of cell cycle progression. The early stage of the G1 phase is regulated by D-type cyclins; in T cells, cyclin D2 is the first to be induced following activation (180). To examine cell cycle progression at the molecular level, we determined whether TCR and CD28 stimulation could independently induce the expression of cyclin D2, in the absence of Jak3-dependent cytokine signals. As shown in Figure 2.6a, CD4 SP thymocytes from *Jak3*$^{-/-}$ OT-II Bcl-2 mice up-regulate cyclin D2 comparably to cells from control mice at day three of activation. Timepoint analysis further confirmed that the level of cyclin D2 increased at 48h and peaked at 96h after activation in both *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 cells (Figure 2.6a, lower panel). Whereas cyclin D2 controls the entry of cells into the G1 phase, passage through the G1 restriction point into the late G1 stage requires the induction of cyclin E. When we examined cyclin E expression, we also saw no difference between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 cells at 48h or 96h post-activation (Figure 2.6a).

To drive cell cycle progression, cyclins associate with cyclin-dependent kinases to form active holoenzymes. These holoenzymes are inhibited by CDKIs; specifically, p27kip1, which is constitutively expressed in resting naïve T cells, inhibits the activities of cyclinD2/cdk4/6 and cyclinE/cdk2 (168). Therefore, cell cycle progression depends on the down-regulation of p27kip1, in addition to the up-regulation of cyclins. When levels of p27kip1 were examined in activated *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 cells, we found that p27kip1 was undetectable in both cell types by 3 days post-stimulation (Figure 2.6a). Finally, we examined cyclin A, which is required for the cells to progress through S

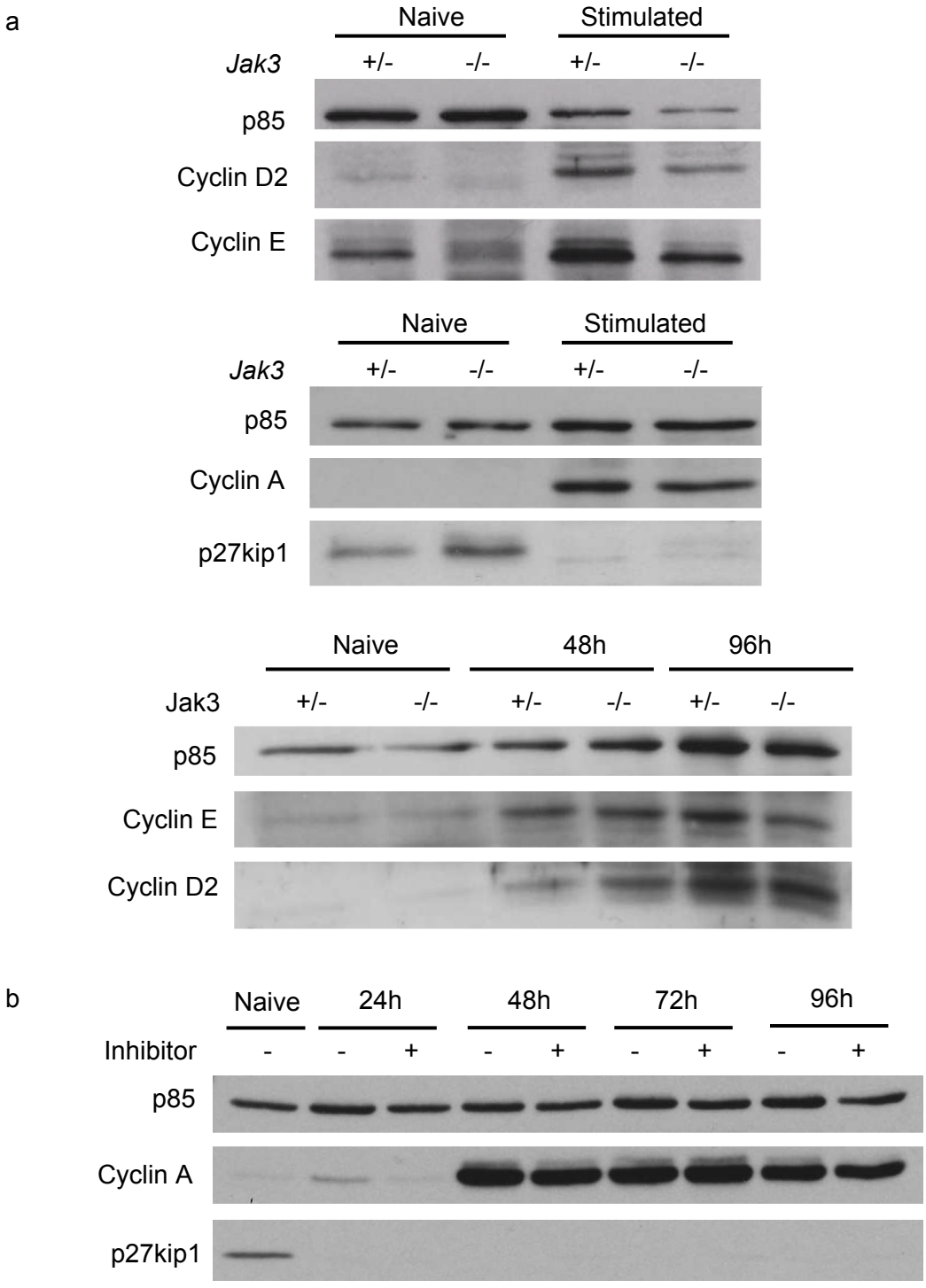
phase. Again, upregulation of cyclin A was comparable between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ T cells (Figure 2.6a). The upregulation of cyclin A and the loss of p27kip1 were confirmed with wild-type T cells stimulated in the presence of the Jak3 inhibitor, PS078507. Furthermore, an extensive timecourse indicated no differences in the kinetics of these changes with or without Jak3-dependent cytokine signals (Figure 2.6b).

Figure 2.6 Cell cycle proteins are regulated normally in the absence of Jak3-dependent cytokine signals.

(a) Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days (upper panel) or for indicated times (lower panel). Total cell lysates were prepared and immunoblotted for p85, cyclin D2, cyclin E, cyclin A and p27kip1. PI-3-kinase p85 was detected as a loading control.

(b) Purified CD4^{+} splenocytes from OT-II-transgenic mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence or absence of PS078507 at 312 nM. At the indicated time points, cells were harvested and total cell lysates were prepared and innunolotted for p85 (loading control), cyclin A and p27kip1.

Figure 2.6


a
Naive
Stimulated
Jak3
+/-
-/-
+/-
-/-
p85
Cyclin D2
Cyclin E
Naive
Stimulated
Jak3
+/-
-/-
+/-
-/-
p85
Cyclin A
p27kip1
Naive
48h
96h
Jak3
+/-
-/-
+/-
-/-
+/-
-/-
p85
Cyclin E
Cyclin D2
b
Naive
24h
48h
72h
96h
Inhibitor
-
-
+
-
+
-
+
-
+
p85
Cyclin A
p27kip1

CD28 costimulation substitutes for cytokine signals to drive T cell proliferation

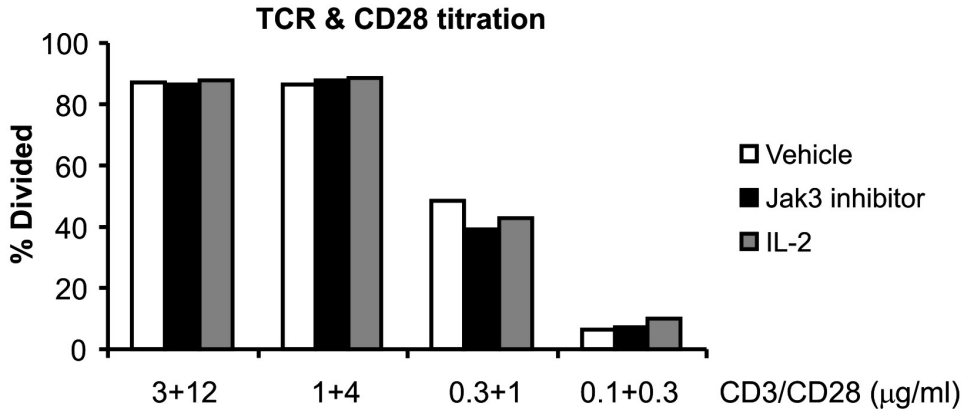
Our findings thus far indicated that Jak3-dependent cytokine signals are not required for T cell proliferation or cell cycle progression. However, it remained possible that cytokine-independent T cell proliferation requires strong TCR and/or CD28 stimulation. To examine whether Jak3-dependent cytokine signals are more essential under conditions of suboptimal T cell stimulation, we performed titration experiments in which the strength of TCR or CD28 stimulation was varied. As the anti-CD3 and anti-CD28 antibodies were titrated down, a substantial reduction in T cell proliferation was observed. The inhibition of Jak3 activity had no effect at any of the conditions tested, nor did addition of exogenous IL-2 (Figure 2.7a). In a second set of experiments, the concentration of anti-CD28 antibody was fixed at 4 μg/ml and the anti-CD3 antibody was varied. As shown in figure 2.7b, T cell proliferation was largely unaffected by this wide range of TCR stimulation conditions, and was also independent of Jak3 signaling. Finally, we fixed the concentration of anti-CD3 antibody at 5 μg/ml and varied the concentration of anti-CD28 antibody. In the absence of the Jak3 inhibitor, CD4+ T cells proliferated robustly in response to each stimulation condition. However, when CD28 stimulation was limiting, inhibition of Jak3-dependent cytokine signaling led to a marked inhibition of T cell proliferation (Figure 2.7c). These results indicate that optimal CD28 stimulation can replace Jak3-dependent cytokine signals to drive T cell proliferation, and further, that when CD28 costimulatory signals, but not TCR signals, are limiting, T cell proliferation becomes cytokine-dependent.

Figure 2.7 Jak3-dependent cytokine signals are required for T cell proliferation when CD28 costimulation is suboptimal.

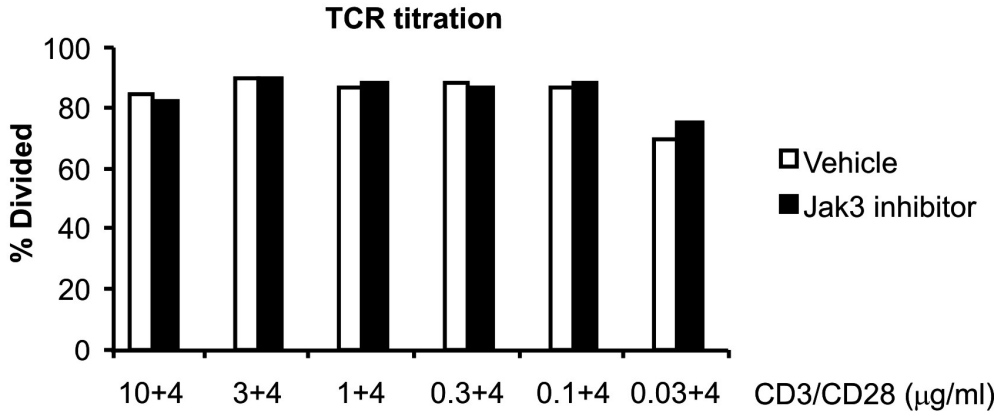
(a, b, c) Purified CD4+ splenocytes from OT-II-transgenic mice were labeled with CFSE and stimulated with indicated concentrations of anti-CD3 and anti-CD28 antibodies in the presence of vehicle alone or PS078507 at 312 nM, and then analyzed by flow cytometry. In (a), recombinant IL-2 was added at 10 ng/ml. Bar graphs show the percentage of T cells that divided without or with the treatment of the inhibitor. Bar graphs represent one of the two independent experiments.

Figure 2.7

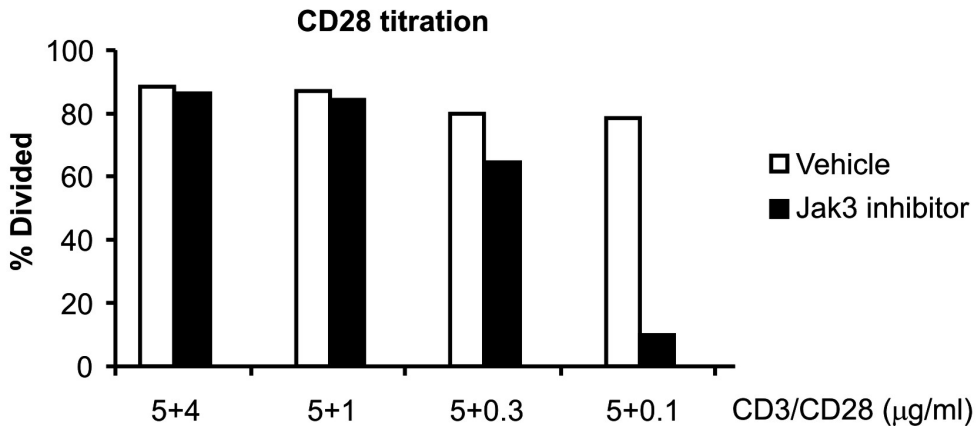
a



b



c



Discussion

Generation of a pool of daughter cells from a small number of naïve T cells is an essential step for the adaptive immune response against pathogens. Following clearance of an infection, the expanded effector cells are eliminated to prevent the pathological accumulation of these potent cells. Thus, both cell proliferation and cell death must be tightly controlled to maintain the integrity of the immune system. In this report, we demonstrate that Jak3-dependent cytokine signals are not required for naïve CD4+ T cell *in vitro* proliferation; instead, they are critical for cell survival. Optimal TCR/CD28 signaling, in the absence of cytokine signals, induces cell cycle progression by modulating cell cycle regulators, such as cyclins and p27kip1. However, we find that under suboptimal stimulation conditions, CD28 signaling is critical in promoting IL-2-independent T cell proliferation.

T cell proliferation is generally accompanied by cell death, an essential aspect of preventing the over-expansion of immune cells. However, during a productive immune response, the proliferating T cells must be temporarily protected from cell death. The role of γc cytokines in promoting the survival of resting T cells, and in maintaining the population of naïve T cells *in vivo*, is well established (129). However, evidence demonstrating that activated T cells can be killed by cytokine withdrawal also points out the key role of cytokine signals, in particular γc cytokine signals, in promoting the survival of activated T cells (181). This latter role of γc cytokine signals does not appear to be mediated solely through regulation of Bcl-2. Although constitutive expression of

Bcl-2 leads to the accumulation of nearly normal numbers of naïve resting $CD4^+$ T cells in $Jak3^{-/-}$ or $\gamma c^{-/-}$ mice (129, 179), Bcl-2 expression only partially reverses the impaired survival of activated $Jak3^{-/-}$ T cells.

These data suggest that γc cytokine signals differentially regulate the survival of naïve versus activated $CD4^+$ T cells. In this regard, a subgroup of Bcl-2 family proteins, the Bcl-2 homology (BH) 3-only proteins, play an essential role in activated T cell death (182, 183), leading to the conclusion that it is the ratio of BH3-only proteins (pro-apoptotic) to Bcl-2-like proteins (anti-apoptotic) that determines whether activated T cells live or die (181). In a second mechnism, the NFκB-regulator/coactivator Bcl-3 has also been implicated in activated T cell death (184), (181). Thus, Jak3-dependent cytokine signals acting in the first several days following T cell activation may prevent the upregulation of BH3-only proteins or induce the expression of Bcl-3.

The issue of whether IL-2 is required for T cell proliferation has been a long-standing controversy. Initial experiments characterizing IL-2 demonstrated that this cytokine functions as a T cell growth factor, leading to the conclusion that IL-2 is crucial for T cell proliferation (147-149). More recent studies clearly indicate that T cells are capable of proliferating in the absence of IL-2R signals. However, the proliferative responses of IL-2- or IL-2R-deficient cells to antigen stimulation are highly variable among experiments. Some experiments show that these responses are only 5-10% of control T cells (161, 185, 186). Other experiments demonstrate that the proliferative responses of T cells lacking

IL-2 signals are 50% of control T cells, and that the replication of these cells is limited to only 2-3 rounds of cell division (164).

In contrast to these earlier findings, our studies indicate that naïve T cell *in vitro* proliferation occurs independently of all γc cytokine signals, including IL-2. Further, we show that T cells lacking IL-2 signals are capable of undergoing at least 7 rounds of cell division *in vitro*. The discrepancy between our results and those reported previously is likely due to two factors. First, the traditional method used to measure T cell proliferation is by tritiated-thymidine incorporation. As this technique cannot distinguish between reduced proliferation and increased cell death, it is likely that the proliferative responses of T cells in the absence of IL-2 were underestimated in earlier studies. Second, some previous studies were performed with mixtures of naïve T cells and activated/memory T cells. Since activated/memory T cells may be more dependent on IL-2 for clonal expansion, this may also have led to conclusions not entirely applicable to naïve CD4+ T cells.

Since IL-2 is not the sole cytokine secreted by activated T cells, we expanded our study to simultaneously assess the role of all γc cytokines in T cell proliferation by targeting the downstream kinase, Jak3, required for all of these receptor signaling pathways. Our results, that naïve CD4+ T cell proliferation is independent of cytokines, are consistent with a recent paper from Di Santo and colleagues, who reported robust *in vitro* proliferation of $\gamma c^{-/-}$ T cells (129). These data indicated that signaling through the TCR

plus CD28 is sufficient to induce T cell proliferation. However, this latter study limited their analysis to examining CFSE profiles of T cells at day 4-6 post-activation. No examination at early timepoints after activation, no cell cycle analysis, no biochemical analysis of cell cycle regulators, and no assessment of the differential roles of TCR versus CD28 signaling were included in this former study. Thus we have extended these previous findings with a more thorough examination of cell cycle progression in the absence of Jak3-dependent cytokine signals, and have also discovered an important role for cytokines when costimulatory signals are limiting.

Past studies using IL-2 and IL-2R blocking antibodies or IL-2-deficient cells, have shown that TCR/CD28 stimulation directly promotes the upregulation of cyclins and the downregulation of p27kip1. However, in these studies it was difficult to determine whether the blocking antibodies were able to completely inhibit IL-2R signaling, and in addition, activated T cells secrete other γc cytokines that may contribute to the regulation of cell cycle proteins. Therefore, we examined this issue using two approaches, each of which leads to complete inhibition of all γc cytokine signaling pathways. Our results indicate that a genetic deficiency in Jak3, or pharmacological inhibition of Jak3, have no effect on cell cycle progression following optimal stimulation of the TCR plus CD28. Upregulation of cyclin D2, cyclin E and cyclin A, as well as downregulation of P27kip1, are indistinguishable in the presence versus the absence of cytokine signaling. This conclusion was also confirmed by a timecourse of cell cycle analysis quantitating the progression of cells through each phase of the cell cycle.

In our system, we analyzed the role of Jak3-dependent signals in $CD4^+$ T cell through *in vitro* proliferation system. In the absence of Jak3-dependent signals, we used a transgene expressing Bcl-2 to rescue the *in vivo* survival defect of naïve $CD4^+$ T cells. It has been shown that over-expression of Bcl-2 might have anti-proliferative function, especially under suboptimal conditions (187). This anti-proliferative affect could confound our studies, although this trangene was used in both *Jak3*$^{-/-}$ and wild type mice. However, under our *in vitro* simulation conditions (anti-CD3 1μg/ml plus anti-CD28 4μg/ml), we did not observe any proliferative difference in $CD4^+$ T cells from wild type and Bcl-2 transgene mice (data not show). Further, analysis of wild type $CD4^+$ T treated with or without a Jak3 inhibitor showed similar results. Therefore, using the Bcl-2 transgene in our system does not affect the *in vitro* proliferation of naïve T cells.

Another potential caveat to our system is that the high concentration of anti-CD3 and anti-CD28 used during *in vitro* priming of T cells may not mimic *in vivo* responses. In order to better understand the physiological relevance, we can examine the proliferation of naïve $CD4^+$ T cells from OTII transgenic mice stimulated with serially diluted OVA peptide presented by antigen-presenting cells. In addition, we can investigate the *in vivo* proliferation of Jak3-deficient cells by adoptively transferring CFSE-labeled *Jak3*$^{-/-}$OTII or *Jak3*$^{+/-}$OTII T cells into congenic B6 recipients and priming the next day with OVA-pulsed bone-marrow-derived DCs. No proliferative difference will be expected in *Jak3*$^{+/-}$ or *Jak3*$^{-/-}$ T cells.

CHAPTER III

Janus-Kinase-3-Dependent Signals Induce Chromatin Remodeling at the Ifng Locus during T Helper 1 Cell Differentiation

Introduction

After antigen stimulation, naïve $CD4^+$ T cells differentiate into one of several functional classes of effector cells; most frequent among these effector subsets are T helper (Th) type 1 (Th1) and Th type 2 (Th2) cells (188). The distinct patterns of cytokine production by activated Th1 and Th2 effector cells are the consequence of differential gene transcription, a process that is regulated at multiple steps (86, 189). The first step involves changes in chromatin accessibility. Subsequent to this, specific transcription factors access the open loci, where they bind to regulatory regions and activate gene transcription. Finally, binding of some transcription factors to their cis-acting regulatory elements directs chromatin-remodeling activity, leading to heritable changes in inducible transcriptional activity.

The signaling pathways that contribute to Th differentiation have been well characterized (189, 190). When naïve $CD4^+$ T cells are initially stimulated, latent transcription factors non-selectively stimulate low amounts of both IFN-γ and IL-4 production (55). In the presence of IL-12, Th1 differentiation is efficiently induced when IFN-γ stimulates Janus family tyrosine kinase (Jak) 1 and Jak2-dependent IFN-γR signaling, leading to the activation of signal transducer and activator of transcription 1 (STAT1), and thereby inducing expression of the Th1-specific transcription factor, T-bet (25). T-bet up-regulates expression of IFN-γ, as well as the IL-12 receptor β2-subunit (IL-12Rβ2) (23, 25). IL-12, acting via Jak2 and Tyk2 to activate STAT4, is not required for initial Th1

differentiation; instead, IL-12 amplifies the Th1 response by augmenting the production of IFN-γ (191). IL-12 signals together with T-bet also act to down-regulate the lineage commitment and cytokine expression of the alternative Th2 subset (23, 192).

Epigenetic regulation of cytokine loci also play an essential role in Th differentiation (189). The first definite evidence of chromatin changes occurring during Th differentiation was reported by Rao and colleagues, who identified T cell lineage-specific DNAase I hypersensitive sites near the *Ifng* gene promoter and enhancer (81). These hypersensitive sites denote regions where chromatin structure has been altered, and they reflect the occupancy of these sequences with specific DNA binding proteins. Others have confirmed the importance of epigenetic changes in the regulation of Th1 and Th2 cytokine gene transcription (1, 193). The chromatin modification most often studied during Th differentiation is histone acetylation (189).

Changes in histone acetylation at the cytokine loci have been well documented during Th differentiation (55, 82, 85, 97). Interestingly, timecourse analysis indicates that the initial increase in histone acetylation at the regulatory regions of both the *Il4* and *Ifng* genes is elicited in response to T cell receptor (TCR) signaling and is independent of the cytokine milieu (55). However, in Th1- or Th2-polarizing conditions, cytokine locus-specific histone acetylation patterns are observed by day 5 post-activation, and are maintained in the differentiated effector cells (55, 82, 84).

STAT4, the transcription factor activated by IL-12 signaling, has been implicated in maintaining histone acetylation at the *Ifng* locus in Th1 cells (85). The Th1-specific transcription factor, T-bet, also promotes histone acetylation, as enforced expression of T-bet induces DNase I hypersensitivity and histone hyperacetylation at the *Ifng* locus in stimulated STAT4-deficient T cells (23, 82). However, the early events that contribute to *Ifng* locus epigenetic modification in Th1 cells are still not completely elucidated.

The Jak1-STAT1 and Jak2-STAT4 signaling pathways are known to be important for Th differentiation. In contrast, a third Jak kinase, Jak3, has not previously been implicated in Th1 differentiation. Jak3 is required for signaling via the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, all of which share a common receptor subunit, γc. These Jak3-dependent cytokines primarily activate STAT5 and are critical for lymphoid generation, maturation, homeostasis and survival (194). While it is not surprising that Jak3 is essential for Th2 differentiation, due to its essential role in IL-4 signaling, little is known about the role of Jak3-dependent cytokine signals in Th1 differentiation. In this study we examined this issue by assessing Th1 differentiation and IFN-γ production by naïve $CD4^+$ T cells from Jak3-deficient and STAT5-deficient mice. We complemented these studies with analysis of wild type $CD4^+$ T cells treated with a pharmacological inhibitor of Jak3. Together, these experiments demonstrated that Jak3 and STAT5-dependent cytokine signals regulate Th1 differentiation by controlling chromatin remodeling at the *Ifng* locus.

Results

IFN-γ production by Th1 cells is greatly reduced in the absence of Jak3

To investigate the potential role of Jak3-dependent cytokine signals in Th1 differentiation, we established an *in vitro* assay using homogenous populations of naïve *Jak3*$^{-/-}$ CD4^{+} T cells. To accomplish this, we first crossed *Jak3*$^{-/-}$ mice to the transgenic line expressing the OT-II TCR (195). Since γc cytokines are required for naïve CD4^{+} T cell survival *in vivo* (125), we also introduced a transgene expressing Bcl-2 (196). The resulting *Jak3*$^{-/-}$ OTII-transgenic Bcl2-transgenic (hereafter referred to as *Jak3*$^{-/-}$ OT-II Bcl2) mice have peripheral CD4^{+} T cells that predominantly exhibit a naïve (CD44^{lo}) phenotype (Figure 3.1). However, to ensure a starting population of naïve cells, we chose to use CD4^{+}CD8^{-} single-positive (CD4 SP) thymocytes from these mice for our experiments.

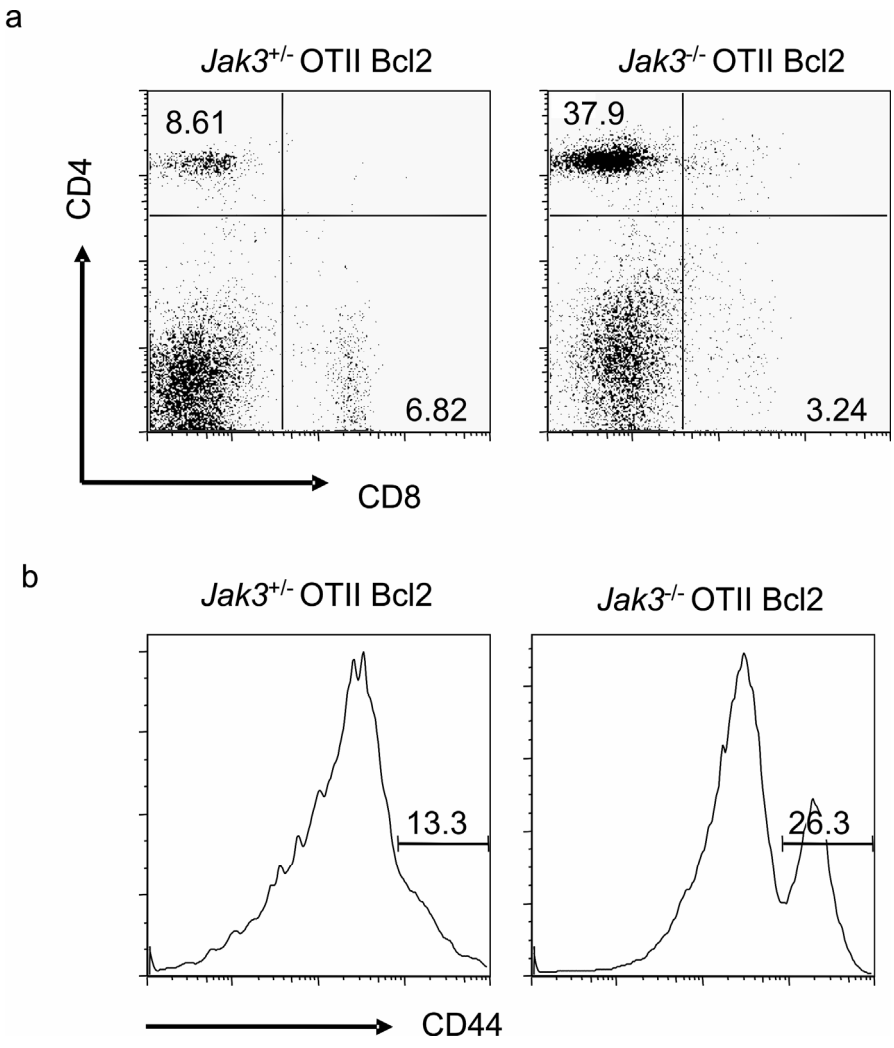
Figure 3.1 CD4+ T cells in Jak3-/- OT-II Bcl2 mice are predominantly naïve.

Splenocytes from the indicated mice were stained with antibodies to CD4, CD8 and CD44, and analyzed by flow cytometry.

(a) Dot-plots show CD4 versus CD8 staining. Numbers indicate the percentages of splenocytes in each quadrant.

(b) Histograms show CD44 profiles on gated $CD4^+$ cells. Numbers indicate the percentage of $CD44^{hi}$ cells in each $CD4^+$ T cell population.

Figure 3.1


a
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
8.61
6.82
37.9
3.24
CD4
CD8
b
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
13.3
26.3
CD44

CD4 SP thymocytes were isolated from *Jak3*$^{+/-}$ or *Jak3*$^{-/-}$ OT-II Bcl2 mice, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), stimulated with anti-CD3 and CD28 antibodies and cultured under non-skewing or Th1-skewing conditions without addition of exogenous IL-2. After four days, cells were restimulated, and IFN-γ was examined by intracellular cytokine staining. As shown in Figure 3.2a, in the absence of Jak3, the percentage of IFN-γ-producing cells was reduced from an average of 12.5±2.7% to 0.5±0.4% ($P < 0.001$) and 27.1±4.1% to 7.0±6.8% ($P < 0.01$) under non-skewing and Th1-skewing conditions, respectively. In addition, the percentage of lymphotoxin α-producing cells was also decreased in the absence of Jak3 (data not shown). Cell division, as assessed by CFSE dilution, was similar between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 cells (3-2A), indicating that the reduced Th1 cytokine production in the Jak3-deficient T cells was not due to impaired proliferation.

To confirm these findings, we examined cytokine secretion by *Jak3*$^{-/-}$ and *Jak3*$^{+/-}$ OT-II Bcl2 T cells following stimulation in Th1- and Th2-polarizing conditions. As shown in Figure 3.2b, under Th1-skewing conditions, *Jak3*$^{-/-}$ CD4 SP cells did not secrete Th2 cytokines (IL-4, IL-5 and IL-10) and produced much less IFN-γ than *Jak3*$^{+/-}$ cells. As expected based on the role for IL-4 receptor signaling during Th2 differentiation (188), *Jak3*$^{-/-}$ CD4 SP cells stimulated under Th2-polarizing conditions did not produce detectable amounts of IL-4, IL-5 or IL-10 (Figure 3.2b).

To determine whether the impaired production of IFN-γ by *Jak3*$^{-/-}$ CD4 SP cells resulted from reduced expression of *Ifng* mRNA, we performed real-time quantitative RT-PCR. Consistent with the protein data, *Ifng* transcript expression was reduced in *Jak3*$^{-/-}$ CD4 SP cells compared to *Jak3*$^{+/-}$ cells, following culture under both non-skewing and Th1-skewing conditions (Figure 3.2c). Together, these data indicate that Jak3-dependent signals are required for optimal production of IFN-γ in differentiated CD4^{+} T cells, and suggest that these signals promote maximal transcription of the *Ifng* gene.

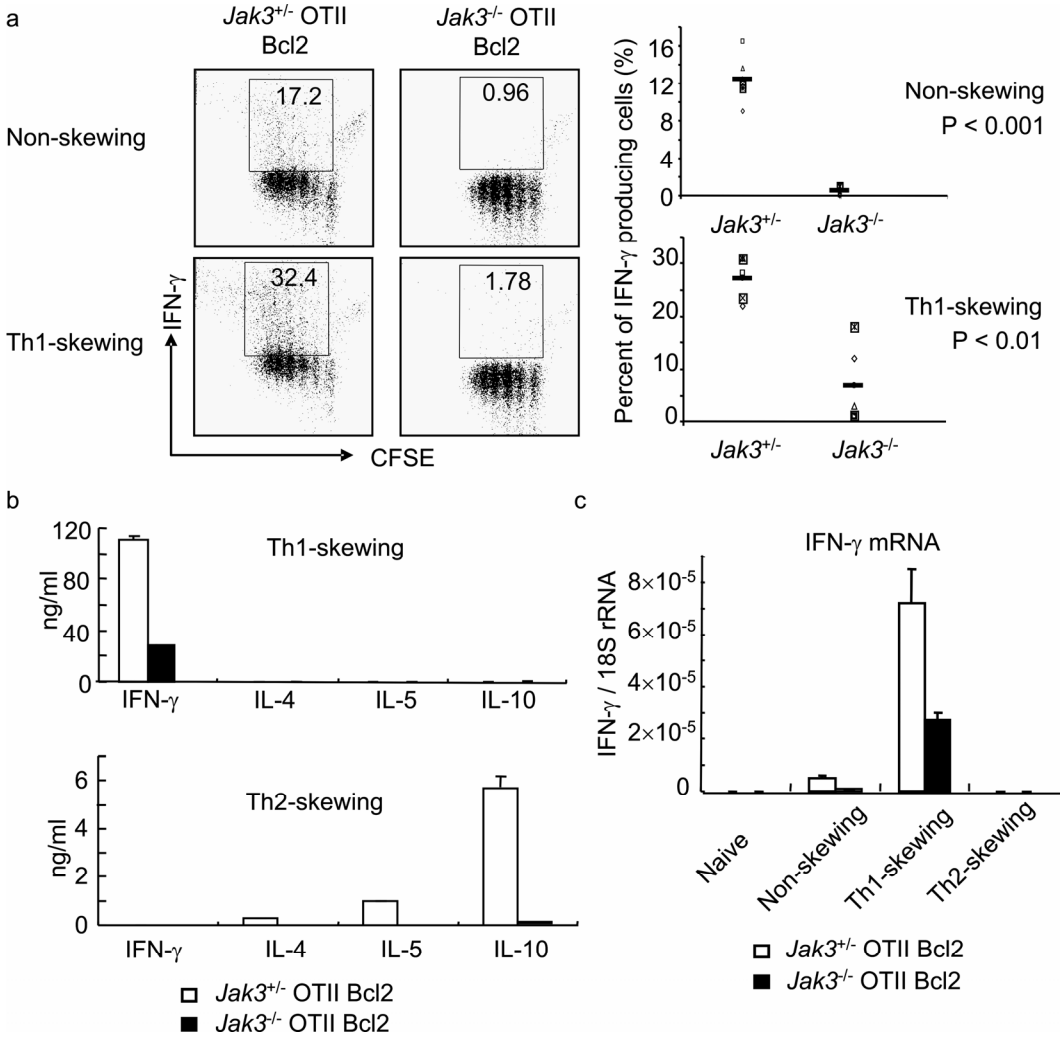
Figure 3.2 Differentiation of CD4+ T cells leads to impaired IFN-γ production in the absence of Jak3.

CFSE-labeled CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated with anti-CD3 plus anti-CD28 and cultured under non-, Th1- or Th2-skewing conditions for 4 days. Non-skewing conditions have no exogenous cytokines added. Th1-skewing conditions have IL-12 and anti-IL-4. Th2-skewing conditons have IL-4 and anti-IFN-γ.

(a) Cells were restimulated with PMA plus ionomycin for 5 hours. Dot-plots show intracellular staining for IFN-γ versus CFSE fluorescence. The graphs show percentages of IFN-γ producing cells from five different experiments, with the means indicated by horizontal bars. Differences between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ responses are statistically significant, with the p values indicated.

(b, c) Following 4-day culture in Th1- or Th2-polarizing conditions, cells were restimulated with PMA and ionomycin for 24h. b) Supernatants were analyzed for production of IFN-γ, IL-4, IL-5 and IL-10 by ELISA. Error bars indicate the standard deviation (SD) of values obtained from stimulations performed in triplicate. c) *Ifng* mRNA expression was analyzed by real-time quantitative PCR. Data are normalized to amounts of 18srRNA in each sample. Error bars indicate the SD of transcript values obtained from triplicate reactions.

Figure 3.2


a
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
Non-skewing
Th1-skewing
17.2
0.96
32.4
1.78
IFN-γ
CFSE
Percent of IFN-γ producing cells (%)
Non-skewing
P < 0.001
Th1-skewing
P < 0.01
Jak3+/-
Jak3-/-
b
Th1-skewing
ng/ml
IFN-γ
IL-4
IL-5
IL-10
Th2-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
c
IFN-γ mRNA
IFN-γ / 18S rRNA
Naive
Non-skewing
Th1-skewing
Th2-skewing

Transcription factors important for Th1 differentiation are independent of Jak3

Our previous studies indicated that T cells from Jak3-deficient mice have intact TCR signaling (197). To examine whether IFN-γ-STAT1 and IL-12-STAT4 pathways are intact in the absence of Jak3, freshly-isolated CD4 SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated with IFN-γ, and assessed for STAT1 tyrosine phosphorylation (Figure 3.3a); further, differentiated *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ Th1 cells were assessed for STAT4 phosphorylation in response to IL-12 stimulation (Figure 3.3b). As shown, both of these responses were intact in the absence of Jak3. We also examined up-regulation of T-bet, a master transcription factor for Th1 differentiation which is induced by IFN-γ signaling in T cells (23, 27). While no T-bet was detected in naïve T cells, *Jak3*$^{+/-}$ CD4 SP cells up-regulated T-bet mRNA and protein after stimulation in both non-skewing and Th1-polarizing conditions (Figure 3.3c and data not shown). *Jak3*$^{-/-}$ cells failed to induce T-bet under non-skewing conditions, perhaps as a result of their failure to produce any IFN-γ following activation. In contrast, *Jak3*$^{-/-}$ cells cultured under Th1-skewing conditions expressed comparable amounts of T-bet mRNA and protein compared to the *Jak3*$^{+/-}$ control cells (Figure 3.3c). Together with our previous studies, these data indicate that three important pathways for Th1 differentiation, TCR signaling, IFN-γ-STAT1, and IL-12-STAT4, are intact in the absence of Jak3-dependent cytokine signals.

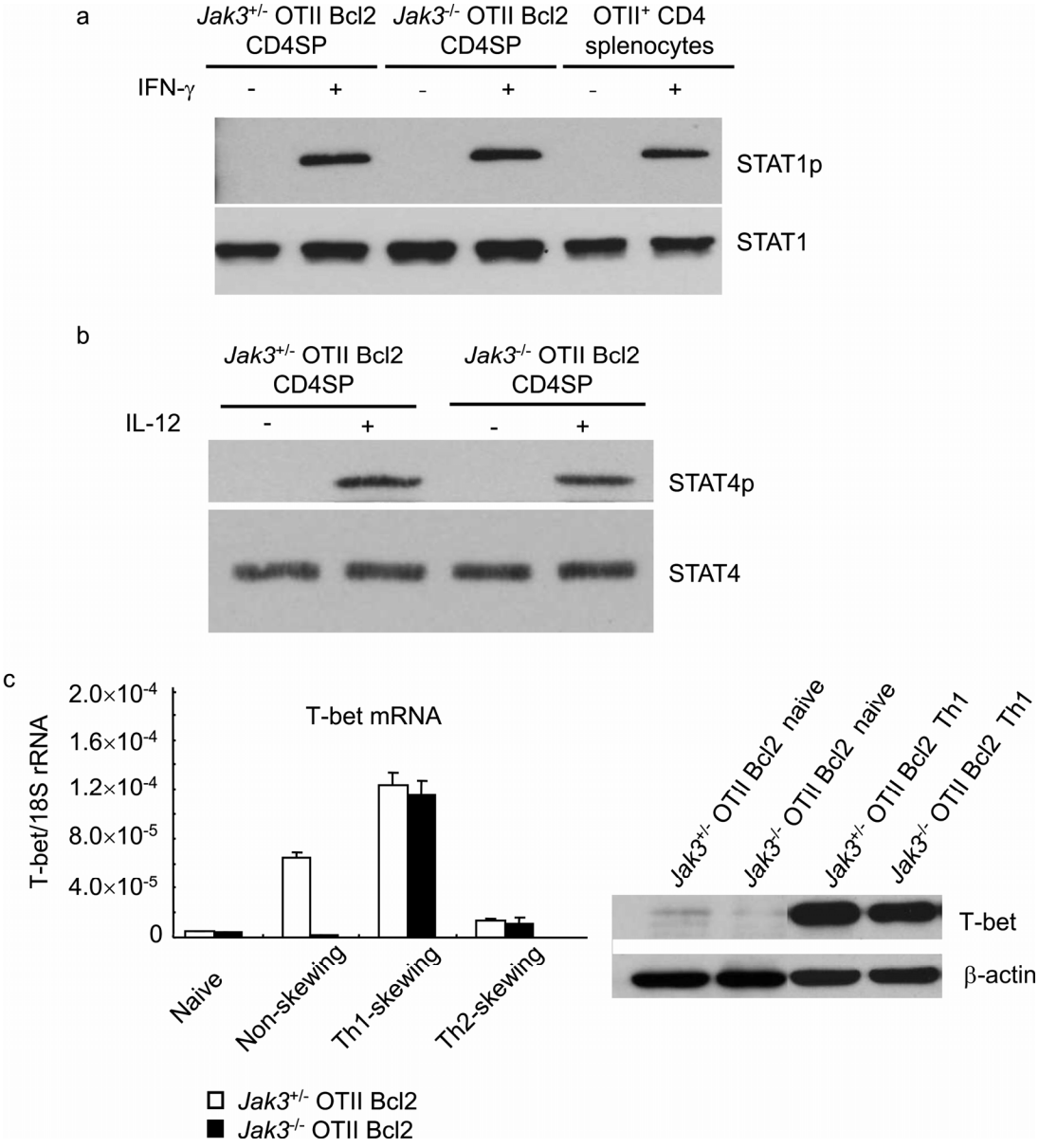
Figure 3.3 STAT1 phosphorylation, STAT4 phosphorylation and T-bet expression are not impaired in Jak3-deficient Th1 cells.

(a) Freshly-isolated purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated with or without IFN-γ (100 ng/ml) for 30 min. Total cell lysates were prepared and blotted for STAT1p and total STAT1. CD4^{+} splenocytes from *Jak3*$^{+/+}$ OTII-transgenic mice were used as a control.

(b) Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were cultured in Th1-skewing conditions for 4 days. Cells were rested overnight, and then stimulated with or without IL-12 (15 ng/ml) for 25 min. Total cell lysates were prepared and blotted for STAT4p and total STAT4.

(c) Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated under non-, Th1- or Th2-skewing conditions for 4 days. T-bet mRNA amounts were analyzed by real-time quantitative PCR. Data are normalized to the levels of 18srRNA in each sample (left panel). Total cell lysates from freshly isolated CD4SP thymocytes (naïve) or Th1-polarized cells (Th1) were prepared and immunoblotted for T-bet and β-actin (right panel). Error bars indicate the SD of transcript values obtained from triplicate reactions.

Figure 3.3


a
Jak3+/- OTII Bcl2 CD4SP
Jak3-/- OTII Bcl2 CD4SP
OTII+ CD4 splenocytes
IFN-γ
- + - + - +
STAT1p
STAT1
b
Jak3+/- OTII Bcl2 CD4SP
Jak3-/- OTII Bcl2 CD4SP
IL-12
- + - +
STAT4p
STAT4
c
T-bet mRNA
T-bet/18S rRNA
2.0×10-4
1.6×10-4
1.2×10-4
8.0×10-5
4.0×10-5
0
Naive
Non-skewing
Th1-skewing
Th2-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
Jak3+/- OTII Bcl2 naive
Jak3-/- OTII Bcl2 naive
Jak3+/- OTII Bcl2 Th1
Jak3-/- OTII Bcl2 Th1
T-bet
β-actin

Pharmacological inhibition of Jak3 leads to impaired IFN-γ production during Th1 differentiation

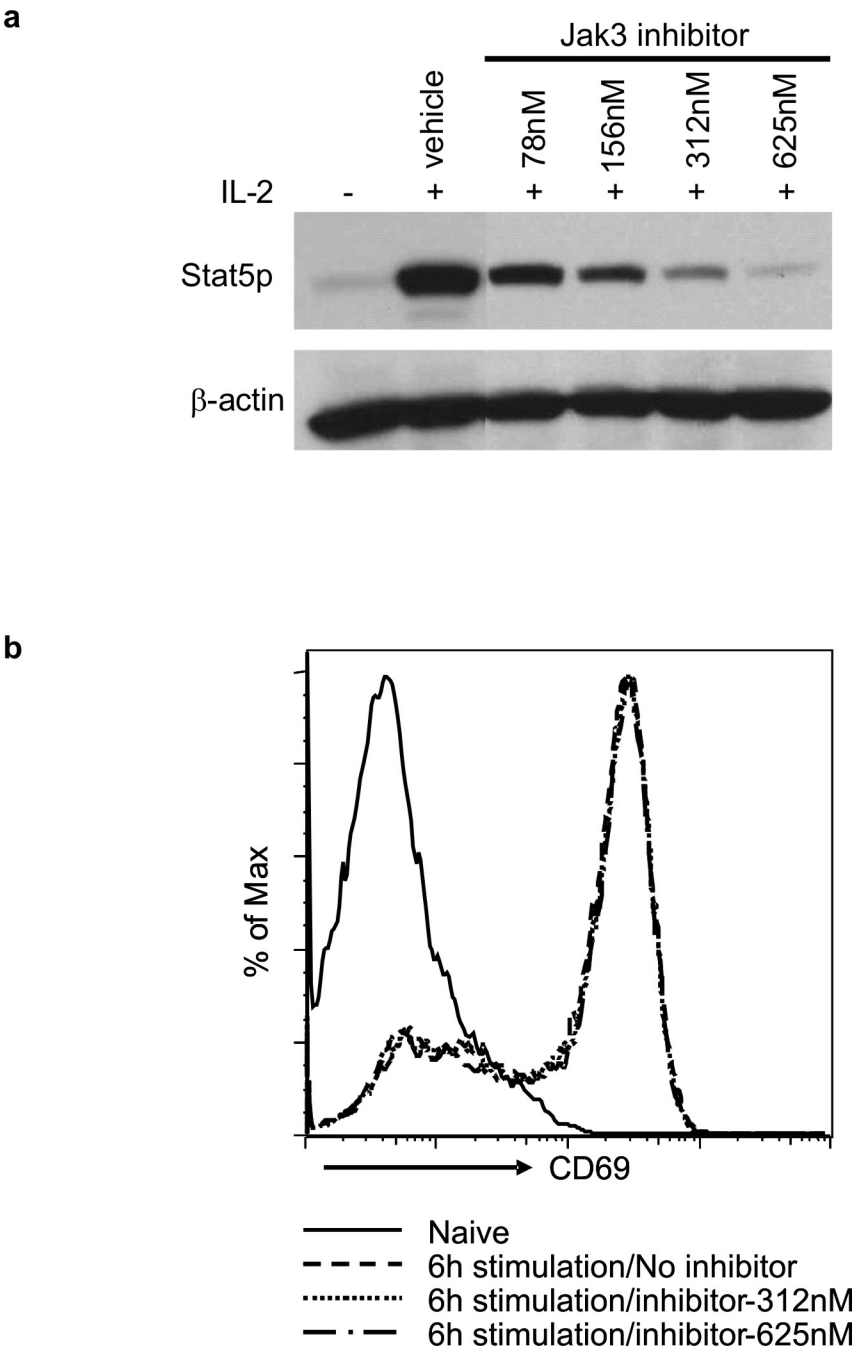
To rule out the possibility that *Jak3*$^{-/-}$ T cells are developmentally abnormal, leading to their impaired Th1 differentiation, we examined cytokine production by *Jak3*$^{+/+}$ CD4^{+} T cells treated with a pharmacological Jak3 inhibitor. To determine the optimal concentration of PS078507 for Jak3 inhibition in murine peripheral CD4^{+} T cells, we assessed IL-2-induced STAT5 phosphorylation after treatment with varying concentrations of PS078507 (Figure 3.4a). PS078507 completely inhibited IL-2-induced STAT5 phosphorylation in *Jak3*$^{+/+}$ CD4^{+} T cells at 625nM. We also found that PS078507 had no effect on T cell proliferation induced by TCR stimulation over the course of a 4-day assay (Figure 3.5a). Furthermore, PS078507 had no inhibitory effect on TCR signaling, as assessed by CD69 up-regulation following TCR stimulation of naïve *Jak3*$^{+/+}$ CD4^{+} T cells (Figure 3.4b).

Figure 3.4 Characterization of the Jak3 inhibitor, PS078507.

(a) Purified $CD4^+$ splenocytes from *Jak3*$^{+/+}$ mice were stimulated with anti-CD3 plus anti-CD28 for 2 days, rested for 4 hours, incubated with vehicle alone or serial-diluted PS078507 for 15 minutes, then stimulated with medium (first lane) or IL-2 (50 ng/ml, all other lanes) for 15 minutes. Cell lysates were prepared and immunoblotted for STAT5p and β-actin.

(b) $CD4^+$ T cells were purified from *Jak3*$^{+/+}$ OTII-transgenic mice and stimulated with anti-CD3 plus anti-CD28 in the presence of vehicle alone, or the Jak3 inhibitor PS078507 at 312 nM or 625 nM. After 6 hours, cells were harvested and stained with anti-CD69 antibodies and analyzed by flow cytometry. Histograms show CD69 expression on unstimulated cells, and cells stimulated under the indicated conditions.

Figure 3.4


a
Jak3 inhibitor
+ vehicle
+ 78nM
+ 156nM
+ 312nM
+ 625nM
IL-2
-
+
Stat5p
β-actin
b
% of Max
CD69
Naive
6h stimulation/No inhibitor
6h stimulation/inhibitor-312nM
6h stimulation/inhibitor-625nM

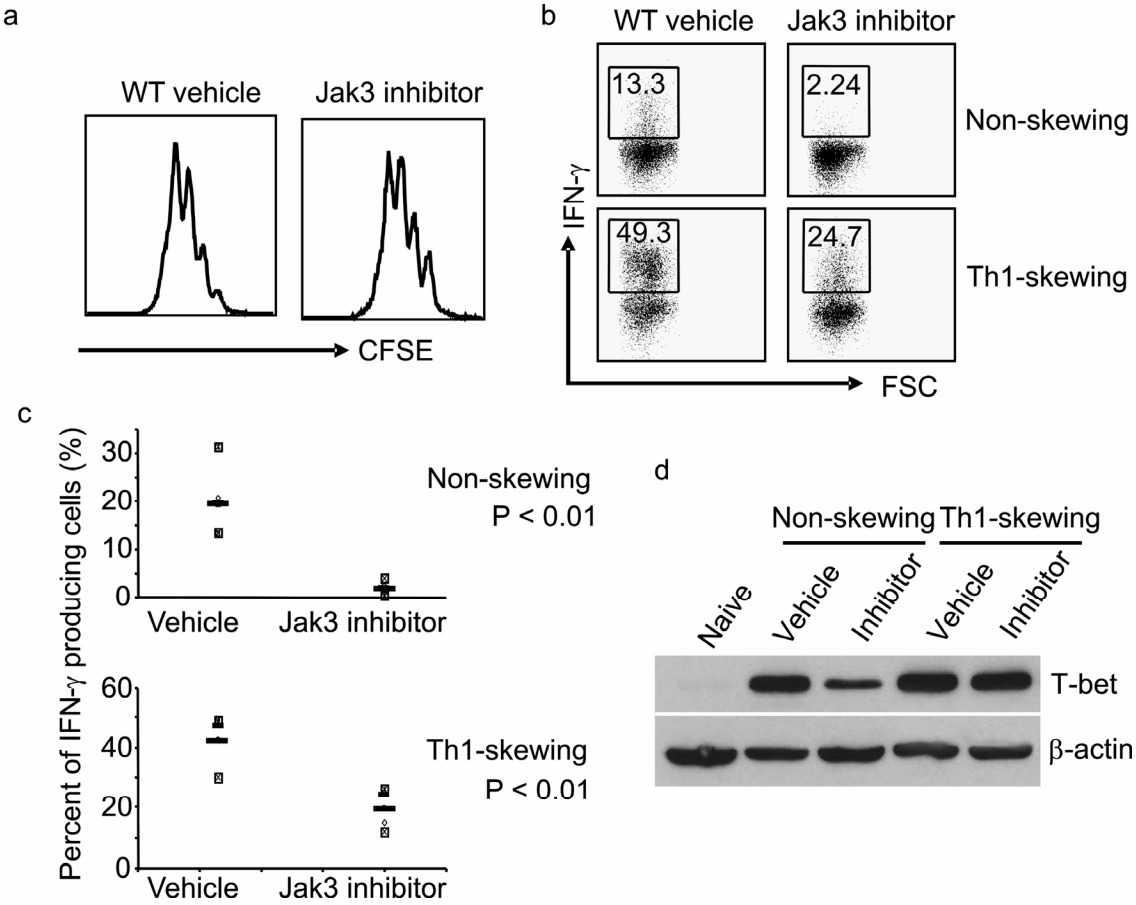
We then tested PS078507 for its effect on Th1 differentiation leading to IFN-γ production. As shown in Figure 3.5b and 3.5c, inhibition of Jak3 enzymatic activity in *Jak3*$^{+/+}$ OT-II transgenic CD4^{+} T cells greatly diminished the percentage of IFN-γ secreting cells from an average of 19.7±7.3% to 1.9±1.3% ($P < 0.01$) under non-skewing conditions, and 42.7±8.9% to 19.5±7.0% ($P < 0.01$) under Th1-skewing conditions. As seen with *Jak3*$^{-/-}$ T cells, T-bet expression was greatly reduced in CD4^{+} T cells cultured under non-skewing conditions in the presence of PS078507; however *Jak3*$^{+/+}$ T cells stimulated under Th1-polarizing conditions showed normal up-regulation of T-bet, even in the presence of the Jak3 inhibitor (Figure 3.5d). These findings confirmed the results seen with *Jak3*$^{-/-}$ CD4 SP cells. Together, these data indicate that the impaired production of IFN-γ in Th1 cells differentiating in the absence of Jak3-dependent signals cannot be explained by defects in the known transcription factors required for *Ifng* gene expression.

Figure 3.5 Pharmacological inhibition of Jak3 during Th1 differentiation leads to impaired IFN-γ production.

(a) Purified $CD4^+$ splenocytes from *Jak3*$^{+/+}$ OTII-transgenic mice were labeled with CFSE and stimulated with anti-CD3 plus anti-CD28 for 4 days in the presence of vehicle alone (WT vehicle) or PS078507 at 625 nM (Jak3 inhibitor). Histograms show CFSE fluorescence.

(b, c, d) $CD4^+$ splenocytes from *Jak3*$^{+/+}$ OTII-transgenic mice were cultured under non- and Th1-skewing conditions for 4 days, in vehicle alone or PS078507 (625 nM). Cells were restimulated with PMA plus ionomycin for 6 hours. b) Dot-plots show IFN-γ intracellular staining versus forward scatter. c) The graphs show percentages of IFN-γ producing cells from four different experiments, with the means indicated by horizontal bars. Differences between cells cultured in vehicle alone or PS078507 (Jak3 inhibitor) are statistically significant, with the p values indicated. d) Total cell lysates were prepared and immunoblotted for T-bet and β-actin.

Figure 3.5


a
WT vehicle
Jak3 inhibitor
CFSE
b
WT vehicle
Jak3 inhibitor
13.3
2.24
49.3
24.7
Non-skewing
Th1-skewing
IFN-γ
FSC
c
Percent of IFN-γ producing cells (%)
30
20
10
0
Vehicle
Jak3 inhibitor
Non-skewing
P < 0.01
60
40
20
0
Vehicle
Jak3 inhibitor
Th1-skewing
P < 0.01
d
Non-skewing
Th1-skewing
Naive
Vehicle
Inhibitor
Vehicle
Inhibitor
T-bet
β-actin

In vivo binding of T-bet to the _Ifng_ promoter is impaired in the absence of Jak3 signals

The *Ifng* gene is a direct target of the transcription factor, T-bet (31). Since T-bet protein expression was not reduced in the absence of Jak3-dependent signals, we considered the possibility that Jak3-dependent signaling might regulate the ability of T-bet to interact with its responsive element in the *Ifng* promoter. To address this, chromatin immunoprecipitation (ChIP) assays were performed to assess the *in vivo* binding of T-bet to the *Ifng* proximal promoter in $Jak3^{+/-}$ versus $Jak3^{-/-}$ OT-II Bcl2 cells (Figure 3.6; primer set #1). CD4 SP thymocytes were activated and cultured under Th1-skewing conditions for 4 days. As shown in Figure 3.7a, naive CD4 SP thymocytes from $Jak3^{+/-}$ or $Jak3^{-/-}$ OT-II Bcl2 mice had no detectable T-bet bound to the *Ifng* promoter (lane 3 and lane 6). Following Th1 differentiation, T-bet bound to the *Ifng* promoter was easily detectable in $Jak3^{+/-}$ T cells, but substantially diminished in $Jak3^{-/-}$ T cells (lane 9 and lane 12). We confirmed these findings with $Jak3^{+/+}$ $CD4^{+}$ T cells stimulated in Th1-skewing conditions in the presence of the Jak3 inhibitor PS078507 (Figure 3.7b). Overall these data correlated with the pattern of IFN-γ production by $Jak3^{-/-}$ T cells, or following Jak3 inhibition, suggesting that reduced production of IFN-γ under these conditions may result from an impaired ability of T-bet to bind to the *Ifng* promoter, and thereby to activate *Ifng* gene transcription.

Figure 3.6 Schematic of the Ifng regulatory regions.

The positions of PCR primers for ChIP experiments and accessibility assay are labeled, and indicated on a map of the murine *Ifng* locus.

Figure 3.6

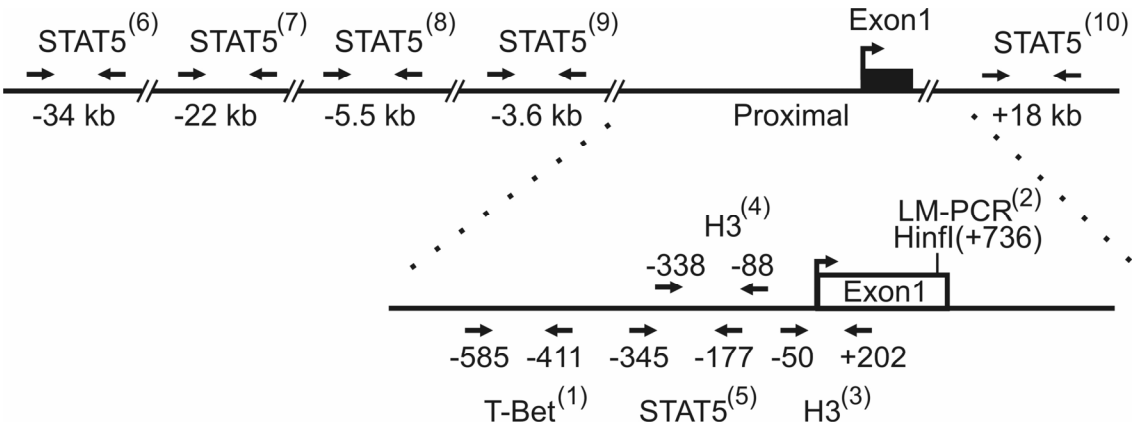
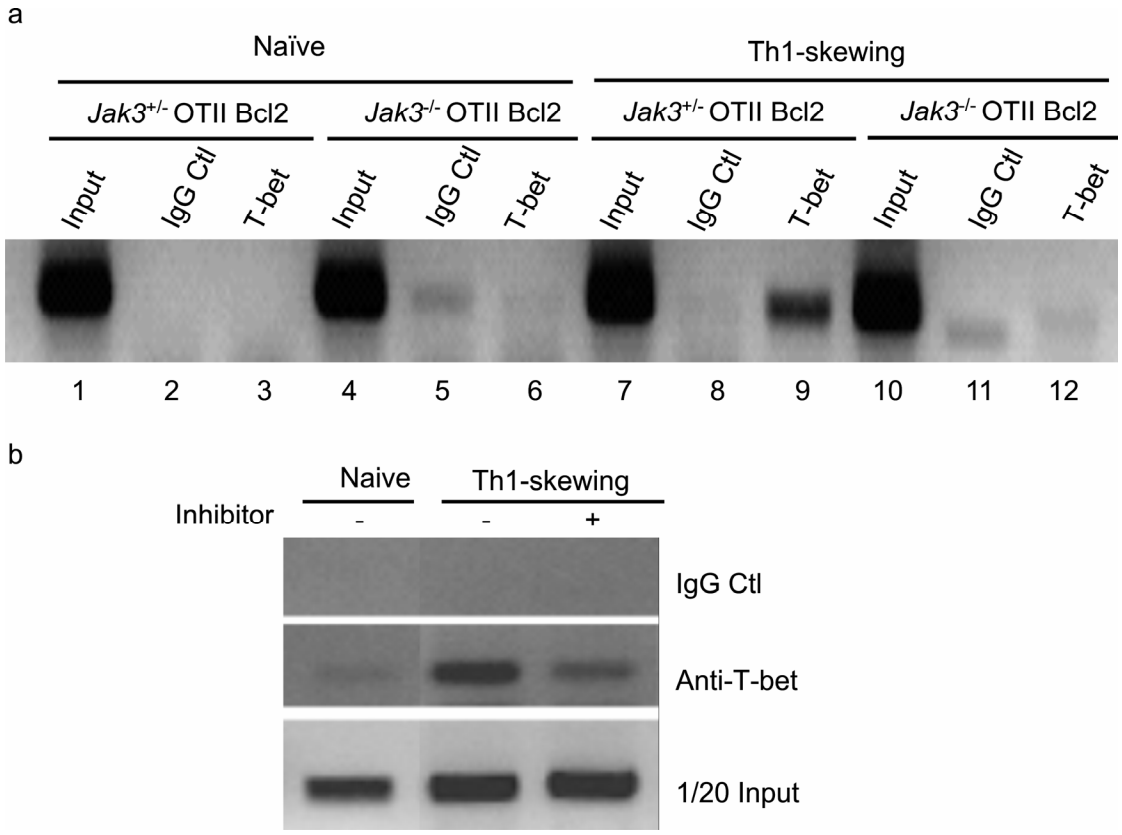

STAT5(6)
STAT5(7)
STAT5(8)
STAT5(9)
Exon1
STAT5(10)
-34 kb
-22 kb
-5.5 kb
-3.6 kb
Proximal
+18 kb
H3(4)
LM-PCR(2)
HinfI(+736)
-338
-88
Exon1
-585
-411
-345
-177
-50
+202
T-Bet(1)
STAT5(5)
H3(3)

Figure 3.7 In vivo binding of T-bet to the Ifng promoter is impaired in Jak3-deficient cells.

(a) Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were cultured under Th1-skewing conditions for 4 days (lanes 7-12) and compared with freshly isolated cells (naïve, lanes 1-6). ChIP analysis was done with an anti-T-bet (lanes 3, 6, 9, 12) or a rabbit IgG control antibody (lanes 2, 5, 8, 11).

(b) CD4^{+} splenocytes from *Jak3*$^{+/+}$ OTII-transgenic mice were cultured with vehicle alone or PS078507 (625nM) under Th1-skewing conditions for 4 days. ChIP analysis was done with a rabbit IgG antibody control (top) or an anti-T-bet (middle). The location of PCR primers is indicated in Figure 3.6.

Figure 3.7

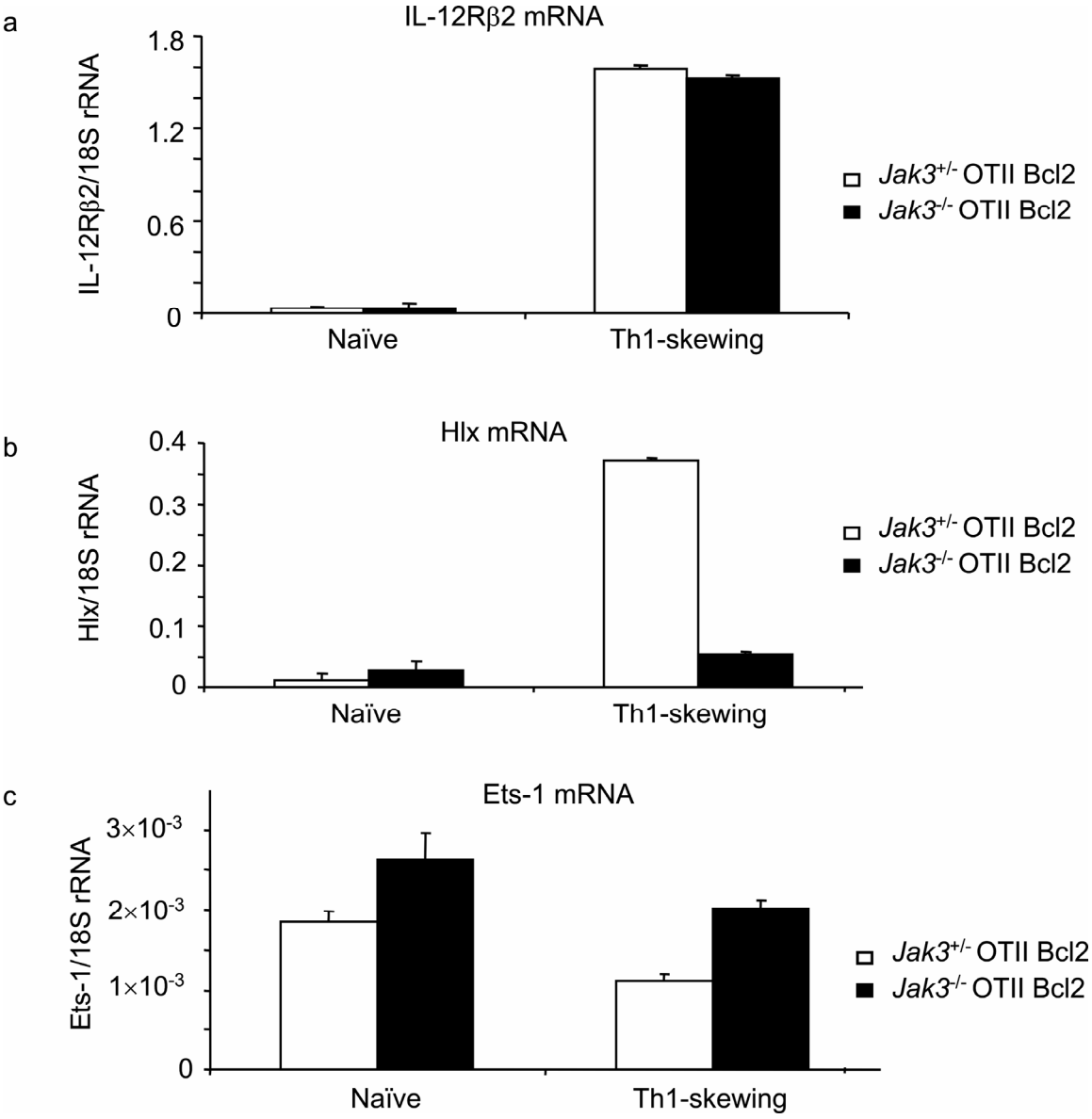

a
Naïve
Th1-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
Input
IgG Ctl
T-bet
1 2 3 4 5 6 7 8 9 10 11 12
b
Naive
Th1-skewing
Inhibitor
- - +
IgG Ctl
Anti-T-bet
1/20 Input

The apparent inability of T-bet to promote transcription of the *Ifng* gene is not due to a global defect in T-bet activity or function. *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 CD4 SP thymocytes were activated and stimulated under Th1-polarizing conditions for 4 days, and mRNA expression of the IL-12Rβ2 chain, Hlx, and Ets-1 genes was examined (Figure 3.8). Both populations showed comparable expression of transcripts encoding the IL-12Rβ2 chain and Ets-1, whereas *Jak3*$^{-/-}$ cells had reduced expression of Hlx mRNA. As IL-12Rβ2 and Hlx are both transcriptional targets of T-bet (23, 26), these findings indicate that T-bet is transcriptionally competent to promote expression of IL-12Rβ2, but selectively loses the ability to mediate transcription of other genes, such as *Ifng* and Hlx.

Figure 3.8 IL-12Rβ2, Hlx and Ets-1 mRNA levels Th1 cells from Jak3$^{+/-}$ and Jak3$^{-/-}$ OT-II Bcl-2 mice.

CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were left unstimulated (naïve) or were stimulated under Th1-skewing conditions for 4 days (Th1-skewing). IL-12Rβ2 mRNA (a), Hlx mRNA (b) and Ets-1 mRNA (c) amounts were analyzed by real-time quantitative PCR. Data are normalized to the amounts of 18SrRNA in each sample.

Figure 3.8


a
IL-12Rβ2 mRNA
IL-12Rβ2/18S rRNA
1.8
1.2
0.6
0
Naïve
Th1-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
b
Hlx mRNA
Hlx/18S rRNA
0.4
0.3
0.2
0.1
0
Naïve
Th1-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
c
Ets-1 mRNA
Ets-1/18S rRNA
3×10-3
2×10-3
1×10-3
0
Naïve
Th1-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2

Th1 cells differentiating in the absence of Jak3 signaling show reduced *Ifng* promoter accessibility

The results described above suggested that T-bet function in Th1 cells may be regulated by chromatin structure. To examine this issue further, we examined the accessibility of the *Ifng* proximal promoter using restriction endonuclease digestion followed by ligation-mediated polymerase chain reaction (LM-PCR; Figure 3.6, primer set #2). As shown in Figure 3.9a, the *Ifng* promoter in naïve *Jak3*$^{+/-}$ or *Jak3*$^{-/-}$ CD4 SP thymocytes was not accessible to endonuclease digestion. Following four days of stimulation under Th1-polarizing conditions, the *Ifng* promoter showed dramatically increased accessibility in *Jak3*$^{+/-}$ SP cells. In contrast, the signal from the *Jak3*$^{-/-}$ SP cells was reduced to ~30% of that seen in the control cells, indicating that the *Ifng* promoter is markedly less accessible in Jak3-deficient T cells cultured under Th1-skewing conditions.

Using the ChIP assay, we next examined the acetylation status of histone H3 at the *Ifng* promoter in Th1 cells stimulated in the presence or absence of functional Jak3 (Figure 3.6, primer set 3). As shown in Figure 3.9b, naïve *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 CD4 SP thymocytes had a low level of histone H3 acetylation at the *Ifng* promoter. Following four days of culture in Th1-skewing conditions *Jak3*$^{+/-}$ cells displayed strong hyperacetylation of histone H3 at the *Ifng* promoter. In contrast, histone H3 acetylation of the *Ifng* promoter was 3-fold reduction in *Jak3*$^{-/-}$ Th1 cells, consistent with the diminished accessibility of this region to restriction endonuclease digestion. These findings were also confirmed with *Jak3*$^{+/+}$ T cells stimulated under Th1-polarizing conditions in the

presence of the Jak3 inhibitor, PS078507 (Figure 3.9c). Furthermore, all findings were confirmed with a second set of PCR primers (Figure 3.6, primer set #4; data not shown). Together, these data strongly suggest that Jak3-dependent cytokine signals are required for chromatin remodeling of the *Ifng* locus during Th1 differentiation.

To further assess the role of histone acetylation in Jak3-dependent IFN-γ production, we pharmacologically-induced histone acetylation of the *Ifng* promoter using a histone deacetylase (HDAC) inhibitor, sodium butyrate. Addition of sodium butyrate during Th1 differentiation of both $Jak3^{+/-}$ and $Jak3^{-/-}$ OT-II Bcl2 CD4 SP thymocytes resulted in increased production of IFN-γ compared to untreated cells (Figure 3.9d). This finding was confirmed with $Jak3^{+/+}$ $CD4^+$ T cells stimulated in the presence of the Jak3 inhibitor, PS078507 (data not shown). Overall, the data presented here indicate that Jak3-dependent cytokine signals induce IFN-γ production via chromatin remodeling of the *Ifng* locus.

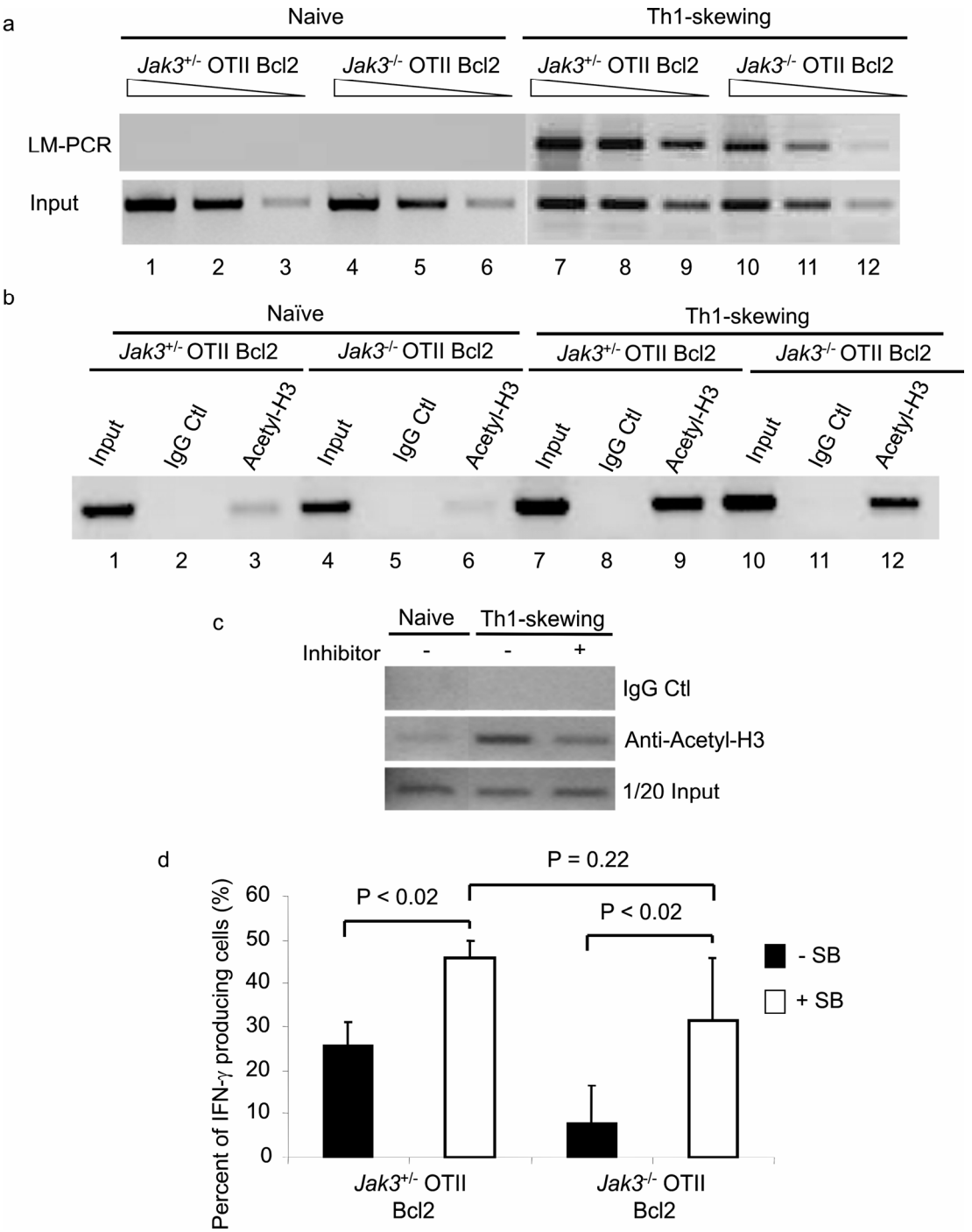
Figure 3.9 Chromatin accessibility of the Ifng promoter is reduced in Th1 cells differentiated in the absence of Jak3-dependent signals.

(a, b) Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated under Th1-skewing conditions for 4 days (lanes 7-12). Freshly-isolated cells (naïve, lanes 1-6) were used for comparison. a) Nuclei were isolated, digested with HinfI, and three-fold dilutions of genomic DNA were subjected to LM-PCR with primers for the *Ifng* proximal promoter (LM-PCR primer set #2). b) ChIP analysis was performed with an antibody to acetylated histone H3 (lanes 3, 6, 9, 12) or a rabbit IgG control antibody (lanes 2, 5, 8, 11) (H3 primer set #3).

(c) CD4^{+} splenocytes from *Jak3*$^{+/+}$ OTII-transgenic mice were cultured in vehicle alone or with the Jak3 inhibitor (625nM) in Th1-skewing conditions for 4 days. ChIP analysis was performed with a rabbit IgG control antibody (top) or antibody to acetylated histone H3 (middle) (H3 primer set #3).

(d) Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated under Th1-skewing conditions. Parallel cultures were treated with or without sodium butyrate (SB; 200μM), added at 24h post-stimulation. At day 4, cells were restimulated with PMA and Ionomycin. The graph shows the percentage of cells producing IFN-γ as assessed by intracellular staining. Data are pooled from three independent experiments. Differences between sodium butyrate treated and untreated samples for each genotype of cells are statistically significant, with p values indicated. Differences between sodium butyrate treated *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ cells are not significant. Error bars indicate the SD of percent of IFN-γ-producing cells obtained from three independent experiments.

Figure 3.9


a
Naive
Th1-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
LM-PCR
Input
1 2 3 4 5 6 7 8 9 10 11 12
b
Naïve
Th1-skewing
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2
Input
IgG Ctl
Acetyl-H3
1 2 3 4 5 6 7 8 9 10 11 12
c
Naive
Th1-skewing
Inhibitor - - +
IgG Ctl
Anti-Acetyl-H3
1/20 Input
d
P = 0.22
P < 0.02
P < 0.02
Percent of IFN-γ producing cells (%)
60
50
40
30
20
10
0
- SB
+ SB
Jak3+/- OTII Bcl2
Jak3-/- OTII Bcl2

Jak3 acts at 24-72 hours following activation to promote histone acetylation at the *Ifng* promoter

Our data indicate that Jak3-dependent signals promote IFN-γ production by inducing histone H3 acetylation. Examining the timecourse of histone H3 acetylation at the *Ifng* promoter during Th1 differentiation by ChIP assay demonstrated only a low basal amount of histone H3 acetylation in the first 24h post-stimulation. Histone H3 acetylation increased at 48h and then increased further at 72h (Figure 3.10a). These data indicate that during Th1 differentiation, selective *Ifng* chromatin remodeling occurs rapidly between 24-72h following activation.

Use of a small molecule Jak3 inhibitor allowed us to assess the kinetic parameters of the Jak3-dependent signal, and to correlate these data to the changes in histone acetylation. For these experiments, *Jak3*$^{+/+}$ CD4^{+} T cells were stimulated under Th1-polarizing conditions in the absence of inhibitor PS078507, or with inhibitor added at varying times following the initial activation of the T cells. At day 4, cells were restimulated and IFN-γ production was examined by intracellular cytokine staining. Consistent with the histone H3 acetylation pattern, PS078507 inhibited IFN-γ production when cells were treated with the inhibitor starting at 0h, 1h, 3h, 6h, 12h and 24h post-stimulation. However, the inhibitory effect of PS078507 was substantially diminished when inhibitor was applied at 48h, and was ineffective when added at 72h post-stimulation (Figure 3.10b). Similarly, acetylation of histone H3 at the *Ifng* promoter was completely inhibited by PS078507 when added 24h after initial T cell activation, but was ineffective when cells were

stimulated under Th1-polarizing conditions for 72h prior to addition of the Jak3 inhibitor (Figure 3.10c). These findings indicate that the predominant action of Jak3-dependent signals in promoting optimal IFN-γ production occurs between 24-72h after stimulation, and that these signals regulate *Ifng* chromatin remodeling during Th1 differentiation.

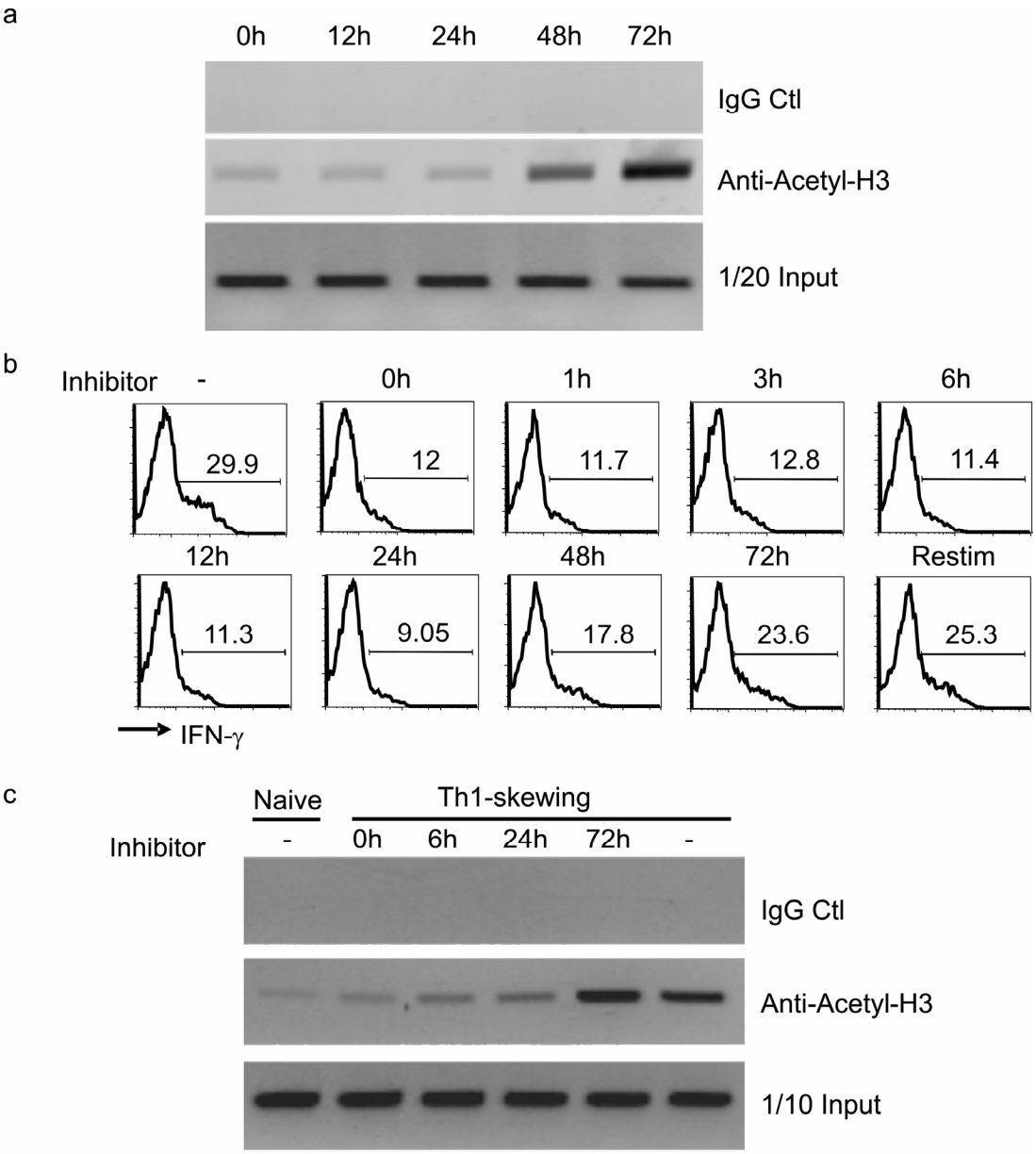
Figure 3.10 The Jak3-dependent signals are required at 24-72h of stimulation to induce histone acetylation at the Ifng locus.

(a) $CD4^+$ splenocytes were isolated from *Jak3*$^{+/+}$ OTII-transgenic mice and were stimulated under Th1-skewing conditions for 0h, 12h, 24h, 48h and 72h. At each timepoint, ChIP analysis was performed with a rabbit IgG control antibody (top) or an antibody to acetylated histone H3 (H3 primer set #3).

(b) $CD4^+$ splenocytes from *Jak3*$^{+/+}$ OTII-transgenic mice were stimulated under Th1-skewing conditions, with vehicle alone or the Jak3 inhbitor (PS078507 at 625nM) added at the indicated timepoints following the initiation of the cultures. All cultures were harvested at day 4, at which time the cells were restimulated and analyzed for IFN-γ production by intracellular cytokine staining. Cell initially cultured in vehicle alone were restimulated in the presence of PS078507 at 625nM (Restim).

(c) $CD4^+$ splenocytes from *Jak3*$^{+/+}$ OTII-transgenic mice were stimulated under Th1-skewing conditions in vehicle alone or with PS078507 (625nM) added at the indicated times following the initiation of the cultures. At day 4, ChIP analysis was performed with a rabbit IgG control antibody (top) or an antibody to acetylated histone H3 (middle) (H3 primer set #3). Freshly isolated *Jak3*$^{+/+}$ OTII-transgenic cells were used for comparison (naïve).

Figure 3.10


a
0h
12h
24h
48h
72h
IgG Ctl
Anti-Acetyl-H3
1/20 Input
b
Inhibitor -
0h
1h
3h
6h
29.9
12
11.7
12.8
11.4
12h
24h
48h
72h
Restim
11.3
9.05
17.8
23.6
25.3
IFN-γ
c
Naive
Th1-skewing
Inhibitor
-
0h
6h
24h
72h
-
IgG Ctl
Anti-Acetyl-H3
1/10 Input

STAT5 and IL-2 promote optimal IFN-γ production during Th1 differentiation

Following Jak kinase activation, STAT5 translocates to the nucleus where it activates the transcription of target genes (198). In human NK cells, a STAT5 binding site has been identified in a distal region of the *IFNG* gene, 3.6kb upstream of the transcriptional start site. This STAT5 binding site serves as a target for epigenetic modification of the *IFNG* locus, or as an IL-2-induced transcriptional enhancer (199, 200). STAT5 also binds to this region of the *IFNG* gene in human T cells, where it enhances *IFNG* gene expression following CD2 stimulation (200). In murine T cells, STAT5 binding to the *Ifng* promoter has not previously been examined. However, several *Ifng* regulatory elements have been identified in these cells through DNase I hypersensitive site (HS) mapping and conserved noncoding sequence (CNS) searching. These elements are *Ifng*CNS-34 (91), *Ifng*CNS-22 (91), *Ifng*CNS-5.5 (90, 95), HS-0.3 (81) and *Ifng*CNS+18 (90), which are -34kb, -22kb, -5.5kb, -0.3kb and +18kb from the IFN-γ transcriptional initiation site, respectively. To investigate whether STAT5 binds to any of these regulatory sites, we utilized the ChIP assay (Figure 3.6, primer sets #5-10). As shown in Figure 3.11a, no STAT5 binding was detected to *Ifng*CNS-34 or *Ifng*CNS-22 (Figure 3.11a, lanes 1 and 2). Furthermore, although STAT5 binds to the -3.6kb region of the human *IFNG* gene, we could not detect STAT5 binding to the corresponding region of the murine *Ifng* locus (Figure 3.11a, lane 4). Interestingly, $CD4^+$ T cells cultured in Th1-polarizing conditions for 48hr exhibited easily detectable STAT5 binding to *Ifng*CNS-5.5 and HS-0.3 following IL-2 stimulation (Figure 3.11a, lanes 3 and 5). In addition, we found extremely weak binding of STAT5 to

*Ifng*CNS+18 (Figure 3.11a, lane 6). These data suggest that the Jak3-STAT5 pathway might directly regulate *Ifng* gene expression.

To determine whether STAT5 is required during Th1 differentiation for optimal IFN-γ production, we utilized T cells from conditional STAT5a and STAT5b double-deficient mice (201). To accomplish this, mice carrying a floxed *Stat5* allele were crossed to CD2-Cre transgenic mice (202). CD4 SP thymocytes were isolated and stimulated under Th1-polarizing conditions. After four days, cells were restimulated and IFN-γ production was assessed by intracellular cytokine staining. Consistent with the data from CD4 SP cells lacking Jak3, STAT5-deficient cells also showed impaired differentiation of IFN-γ-producing Th1 effector cells (Figure 3.11b). Furthermore, addition of sodium butyrate during the differentiation process promoted greatly enhanced production of IFN-γ in T cells lacking STAT5 (Figure 3.11b).

To determine if IL-2 is the cytokine responsible for activating Jak3 and STAT5 during Th1 differentiation and thereby promoting IFN-γ production, we stimulated wild-type (*Jak3*$^{+/+}$ *Stat5ab*$^{+/+}$) T cells under Th1-polarizing conditions in the presence of anti-IL-2 and anti-IL2 receptor blocking antibodies. As shown in Figure 3.11c, inhibition of IL-2 signaling was nearly as effective at preventing the differentiation of IFN-γ-producing Th1 cells as was the Jak3 inhibitor, PS078507. We also tested whether addition of exogenous IL-7 could replace IL-2 and promote optimal IFN-γ production. As shown in Figure 3.11c, IL-7 was only modestly effective at promoting IFN-γ production. The relative

activities of IL-2 and IL-7 at inducing IFN-γ production during Th1 differentiation correlated well with their ability to induce STAT5 tyrosine phosphorylation in differentiating Th1 cells (Figure 3.11d). These data provide further evidence consistent with a role for STAT5 in promoting IFN-γ production during Th1 differentiation.

Figure 3.11 STAT5 and IL-2 are required for optimal IFN-γ production during Th1 differentiation.

(a) Purified $CD4^+$ T cells from *Jak3*$^{+/+}$ OTII-transgenic mice were stimulated and cultured in Th1-skewing conditions for 2 days. Cells were rested overnight, and then restimulated with IL-2 (50 ng/ml) for 1h. ChIP analysis was performed using an STAT5a antibody. Rabbit IgG antibody was used as a negative control. Primers for each of the indicated *Ifng* regulatory regions were designed to detect STAT5 binding (STAT5 primer sets #5-10).

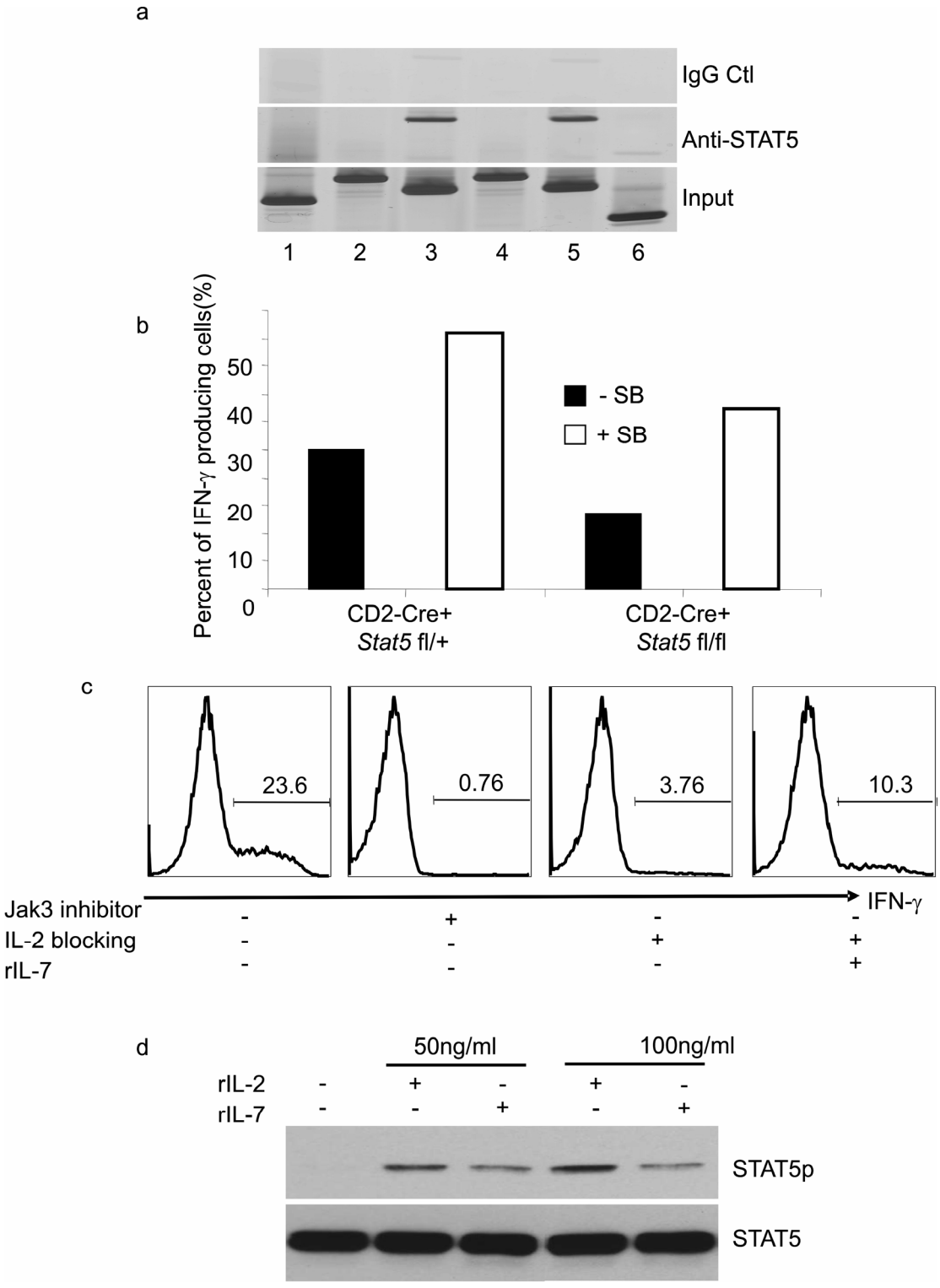
(b) Purified CD4SP thymocytes from CD2-Cre transgenic *Stat5*$^{fl/+}$ and *Stat5*$^{fl/fl}$ mice were stimulated under Th1-skewing conditions. Sodium butyrate (200 μM) was added at 24h post-stimulation. At day 4, cells were restimulated with PMA and Ionomycin. Bar graphs indicate the percentages of IFN-γ producing cells. Data are representative of two independent experiments with similar results.

(c) $CD4^+$ splenocytes from Jak3$^{+/+}$ mice were cultured under Th1-skewing conditions for 4 days, in the indicated conditions. Jak3 inhibitor PS078507 was used at 625nM, IL-2 blocking included anti-IL-2 (10 μg/ml), anti-CD25 (10 μg/ml) and anti-CD122 (10 μg/ml), recombinant IL-7 (rIL-7, 10 ng/ml) was added together with IL-2 blocking antibodies. Cells were restimulated with PMA plus ionomycin for 6 hours. Histograms show IFN-γ intracellular staining.

(d) Purified $CD4^+$ splenocytes from *Jak3*$^{+/+}$ mice were stimulated with anti-CD3 plus anti-CD28 for 24h, rested for 4 hours, then stimulated with medium (first lane), IL-2 or

IL-7, at 50 ng/ml or 100 ng/ml for 15 minutes. Cell lysates were prepared and immunoblotted for STAT5p and total STAT5.

Figure 3.11


a
IgG Ctl
Anti-STAT5
Input
1 2 3 4 5 6
b
Percent of IFN-γ producing cells(%)
0 10 20 30 40 50
- SB
+ SB
CD2-Cre+ Stat5 fl/+
CD2-Cre+ Stat5 fl/fl
c
23.6
0.76
3.76
10.3
IFN-γ
Jak3 inhibitor - + - -
IL-2 blocking - - + +
rIL-7 - - - +
d
50ng/ml
100ng/ml
rIL-2 - + - + -
rIL-7 - - + - +
STAT5p
STAT5

Discussion

Th1 versus Th2 cell fate determination and maintenance are regulated by exogenous signals through the TCR and cytokine receptors, which together activate distinct transcription factor networks and promote epigenetic modifications of lineage-specific cytokine loci (86). Here we show that $CD4^+$ T cells require Jak3-dependent signals to produce optimal amounts of IFN-γ during Th1 differentiation, independently of effects on cell division. Jak3 activity is not required for TCR signaling, nor for the IFN-γ-STAT1-T-bet and IL-12-STAT4 pathways. Instead, Jak3-dependent signals regulate chromatin remodeling at the *Ifng* locus, promote histone H3 acetylation, and control the accessibility of T-bet to the *Ifng* promoter to induce optimal IFN-γ production by Th1 cells.

Helper T cell differentiation is coupled to cell cycle progression. As seen first by Bird and colleagues, IFN-γ is expressed by an increasing frequency of cells with each cell division (1), a finding also illustrated in our data (Figure 3.2a). Although γc-dependent cytokines have been shown to promote lymphoid proliferation (146), we find that cells lacking Jak3 (i.e., $Jak3^{-/-}$) as well as $Jak3^{+/+}$ cells treated with a small molecule Jak3 inhibitor, are able to proliferate comparably to wild type cells in the context of a 4-day *in vitro* assay. Thus we conclude that Jak3-dependent signals are directly influencing effector T cell differentiation, rather than proliferation.

Our data indicate that a third cytokine signal, mediated by Jak3 and STAT5, is required during the early stages of Th1 differentiation (prior to 72h). We complemented our studies on Jak3-deficient and STAT5-deficient T cells with experiments on wild type $CD4^+$ T cells treated with a small molecule inhibitor of Jak3 enzymatic activity, PS078507. In all cases, the inhibitor data are completely concordant with the results obtained using *Jak3*$^{-/-}$ cells, demonstrating that the effects seen are not due to abnormal development of, or compensatory changes in *Jak3*$^{-/-}$ or *Stat5ab*$^{-/-}$ cells. Further, the experiments utilizing the Jak3 inhibitor indicate that Jak3 kinase activity is required for its role in promoting *Ifng* chromatin remodeling.

One potential caveat of the inhibitor experiments is the fact that PS078507 has significant inhibitory activity on Jak1, as well as Jak3. Since Jak1 is a key component of IFN-γ receptor signaling (203), and IFN-γ receptor signaling has been implicated in the induction of T-bet expression and thus in Th1 differentiation (25), inhibition of Jak1 activity by PS078507 could contribute to the impaired Th1 differentiation seen in the presence of the inhibitor. However, several lines of evidence indicate that this scenario is unlikely. First, we detect normal amounts of T-bet in wild-type $CD4^+$ T cells cultured under Th1-polarizing conditions in the presence of PS078507. Second, we observe that IFN-γ receptor is totally absent from $CD4^+$ T cells by six hours after initial activation, and further, that IFN-γ-induced STAT1 phosphorylation is also undetectable by three hours after T cell activation (204, 205) (Figure 3.12). Because we find that PS078507 still inhibits optimal IFN-γ production when added to Th1-polarizing cultures after 24 hours

of activation, it is extremely unlikely that its ability to block this response is due to inhibition of IFN-γ receptor signaling. Thus, the concordance between our findings based on analysis of *Jak3*$^{-/-}$ T cells with the data using PS078507 provide strong support for a role of Jak3 signaling in Th1 differentiation.

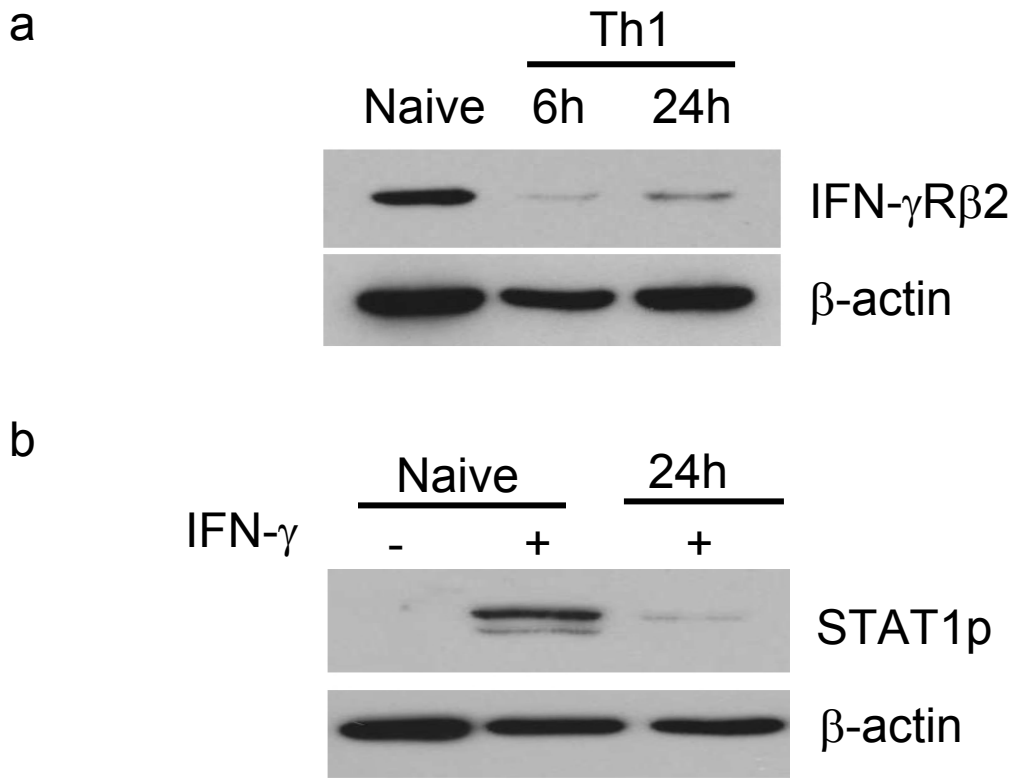
Figure 3.12 IFN-γR β2 and STAT1 phosphorylation are down-regualted in the early stages of Th1 differentiation.

$CD4^+$ T cells were purified from *Jak3*$^{+/+}$ OTII-transgenic mice and stimulated under Th1-skewing conditions.

(a) Following 6h and 24h stimulation, activated T cells were harvested, total cell lysates were prepared and blotted for IFN-γR β2 and β-actin. Naïve $CD4^+$ T cells were used as a control.

(b) Naïve $CD4^+$ T cells and 24h-stimulated Th1cells were stimulated with or without IFN-γ (100 ng/ml) for 30 min. Total cell lysates were prepared and blotted for STAT1p and β-actin.

Figure 3.12


a
Th1
Naive
6h
24h
IFN-γRβ2
β-actin
b
Naive
24h
IFN-γ
-
+
+
STAT1p
β-actin

T-bet is considered the master regulator of Th1 lineage commitment (27). T-bet controls Th1 programs by promoting *Ifng* gene remodeling (23); in addition, T-bet acts to directly induce transcription of the *Ifng* (31), IL-12Rβ2 (23), and Hlx (26) genes. With regard to *Ifng* expression, both of these functions require T-bet binding to the *Ifng* locus. Interestingly, our data indicate that T-bet protein expression alone is not sufficient for maximal T-bet binding to the *Ifng* promoter. These findings could be accounted for by two possible mechanisms. First, it is formally possible that a potential post-translational modification of T-bet (206) is impaired in the absence of Jak3-dependent signals. Arguing against this possibility, the expression of the IL-12Rβ2 chain, one of the direct transcriptional targets of T-bet (23), is not impaired in Th1 cells lacking Jak3, indicating that the T-bet protein present in these cells is transcriptionally active.

Instead, we favor the notion that impaired T-bet binding *in vivo* to the *Ifng* gene is due to reduced accessibility of the *Ifng* locus in the absence of Jak3-dependent signals. In previous studies, the importance of T-bet and STAT4 in *Ifng* chromatin remodeling was demonstrated using T-bet- or STAT4-deficient T cells, or by a retroviral over-expression system (23, 82, 85); therefore it was not possible to determine precisely when these factors carry out their functions. By using a pharmacological Jak3 inhibitor we find that Jak3-dependent signals function at 24-72h following initial T cell activation. Since TCR signals act within 24h to regulate *Ifng* epigenetic modification (55), these data suggest that Jak3-dependent signals function after TCR signaling to promote chromatin remodeling. The impaired ability of T-bet to bind to the *Ifng* promoter in *Jak3*$^{-/-}$ cells

further suggests that Jak3-dependent signals act prior to, or synergistically with T-bet, to regulate *Ifng* gene expression. After 72h, this Jak3-dependent signal is dispensable, and T-bet plus STAT4 are sufficient to reinforce and maintain the open status of the *Ifng* gene.

Chromatin remodeling at the *Ifng* locus may occur by STAT5 recruitment of histone acetyltransferases or chromatin remodeling factors, as has been described in other systems (122, 136, 142-144). While we have been unable to detect constitutive STAT5 binding to the *Ifng* locus in differentiating Th1 cells, IL-2 stimulation of T cells 48h after initial activation induces robust binding of STAT5 to *Ifng* regulatory regions, including *Ifng*CNS-5.5 and HS-0.3. Interestingly, T-bet is also able to bind to these regions (90, 95). As our data indicate that T-bet binding to the *Ifng* proximal promoter is greatly reduced in the absence of STAT5 activation, we propose that STAT5 functions either before, or synergistically with T-bet, to regulate the chromatin remodeling process.

Early studies provided evidence that IL-2 'primes' T cells for production of both IFN-γ and IL-4 (207). More recently, IL-2 receptor signaling via STAT5 has been implicated in the regulation of IL-4 expression and Th2 differentiation, independently of the IL-4/STAT6 pathway (73, 74). These data, together with our findings, suggest the intriguing possibility that IL-2, or another γc-dependent cytokine, may be required in the initial stages of both Th1 and Th2 differentiation for chromatin remodeling at the *Ifng* and *Il4* locus, respectively. This requirement would thereby couple effector $CD4^+$ T cell

differentiation with appropriate survival and growth-promoting signals. In our *in vitro* system, IL-2 is the cytokine activating Jak3 and promoting IFN-γ production during Th1 differentiation. It remains to be determined whether STAT5 is inducing chromatin remodeling at the *Il4* locus during Th2 differentiation. Nonetheless, these data demonstrate that optimal effector $CD4^+$ T cell differentiation depends on an additional cytokine signal that is not lineage-specific, but is required for epigenetic regulation of lineage-specific cytokine loci.

CHAPTER IV

Discussion

A synopsis

The cell-mediated immune response is essential for the immune system to defend against numerous pathogens. Understanding the cellular and molecular regulation of T cell immunity is the basis for developing new drugs that control disease processes. The data presented in this thesis have clearly demonstrated an important role for a group of cytokines, which share the γc chain of their receptors, in the generation and maintenance of T cell effector function.

Whether γc cytokines are essential for T cell proliferation is under debate. In chapter two, I examined the role of γc cytokines during naïve $CD4^+$ T cell proliferation *in vitro*. By inactivating Jak3, the downstream master kinase of γc cytokines, we find that γc cytokines are not required for naïve $CD4^+$ T cell proliferation *in vitro*; rather, they are important for preventing cell death after activation. TCR and CD28 stimulation provides sufficient signals to promote cell cycle progression by up-regulating cyclin D, cyclin E and cyclin A and down-regulating the cell cycle inhibitor, p27kip1. Our data clearly demonstrate that after activation, γc cytokines serve as a survival factor rather than a proliferative factor for $CD4^+$ T cells.

In chapter three, I investigated the function of γc cytokines in naïve $CD4^+$ T cell differentiation. We report that in the absence of Jak3-dependent signals, naïve $CD4^+$ T cells proliferate robustly, but produce little IFN-γ after Th1 polarization *in vitro*. We find

that the defect in IFN-γ production is not due to reduced STAT1 activation in response to IFN-γ signaling, nor is it due to reduced STAT4 activation in response to IL-12. Further, following activation in Th1-skewing conditions, naïve Jak3-deficient $CD4^+$ T cells up-regulate T-bet to a level comparable with wild-type T cells. A chromatin immunoprecipitation assay reveals, instead, that T-bet binding to the *Ifng* promoter is greatly diminished in the absence of Jak3-dependent signals, and that reduced T-bet binding correlates with a decrease in promoter accessibility and histone acetylation at the *Ifng* gene locus. We also show that Jak3-dependent signals function at 24-72h following initial T cell activation to promote histone acetylation at the *Ifng* promoter. To our knowledge, these data provide the first evidence for a critical role of Jak3-dependent cytokine signals in Th1 differentiation, and indicate that Jak3 regulates epigenetic modification and chromatin remodeling of the *Ifng* locus during Th1 differentiation.

Overall, my thesis extends the general knowledge of the role of γc cytokines in the maintenance and function of the immune system. Specifically, we demonstrate that while γc cytokines are not required for T cell proliferation, they are critical for the differentiation and survival of effector T cells. We have discovered a novel signaling pathway for γc cytokines in regulating chromatin remodeling at the *Ifng* locus during Th1 differentiation.

The following sections attempt to reconcile the role of γc cytokines in the effector functions of T cells and propose several models based on our data and the exiting literature.

Proliferation versus differentiation of naïve $CD4^+$ T cells

Naïve $CD4^+$ T cells are activated through the ligation of their TCR and engagement of the costimulatory molecules during antigen presentation by dendritic cells. After activation, $CD4^+$ T cells proliferate and differentiate into three main types of Th cells with distinct cytokine secretion profiles and unique functional characteristics. These cells are referred to as Th1, Th2 and Th17 cells. Th1 cells produce IFN-γ and TNF-β, Th2 cells secrete IL-4, IL-5, IL-6 and IL-10, while Th17 cells produce IL-17. A number of factors are involved in determining the differentiation process, among which cytokines are the most influential factors. Specifically, IL-12 and IFN-γ are important for Th1 differentiation; IL-4 is critical for Th2 differentiation, while IL-6, IL-21 and TGF-β are essential for Th17 differentiation. In addition to effector cells, naïve $CD4^+$ T cells are able to differentiate into several subsets of Treg cells, including induced Tregs (iTregs) and Tr1. Natural occurring Treg (nTreg) cells are generated from the thymic $CD4^+$ T cell precursors. Tr1 cells produce high levels of IL-10 and are generated in the presence of immunosuppressive factors such as IL-10 or vitamin D3 dexamethasone (208).

As presented previously, $CD4^+$ T cells proliferate robustly in the absence of Jak3, which mediates signals for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. However, γc cytokines seem to be essential for the differentiation of these activated cells into all three subsets of Th cells as well as Treg cells. First, as shown above, in the absence of γc-dependent cytokine signals, naïve T cells cannot differentiate into Th1 cells due to a dramatic decrease in IFN-γ production. Second, Paul and colleagues show that STAT5-deficient T cells do not secrete IL-4 or differentiate into Th2 cells when cultured under Th2-skewing conditions (73). Third, we find that the Th17-skewing cytokine environment is incapable of driving the differentiation of Jak3-deficient naïve $CD4^+$ T cells into Th17 cells and that the production of IL-17 is significantly diminished without Jak3-dependent cytokine signals (Figure 4.1). Fourth, our previous work reports that naturally occurring Treg cells fail to be generated in Jak3-deficient mice. However, peripheral $CD4^+$ T cells in Jak3-deficient mice have several characteristics of Tr1 cells. For instance, Jak3-deficient $CD4^+$ T cells produce high levels of IL-10 and modestly inhibit the proliferation of wild type naive $CD4^+$ T cell *in vitro* (128). Whether these cells prevent autoimmune diseases and control T cell number *in vivo* needs to be further elucidated. Finally, it is interesting to see that there is substantially more IL-2 production in Jak3-deficient cells cultured under either non-skewing or Th1-skewing conditions (Figure 4.2), indicating that Jak3-deficient cells stay in Th0 stage rather than differentiate into effector cells.

Figure 4.1 Th17 differentiation is impaired in Jak3-deficient cells.

CD4SP thymocytes were isolated from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice, stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) and cultured under Th17-skewing conditions (IL-6 20 ng/ml, TGF-β 2 ng/ml and anti-IFN-γ 10 μg/ml) for 4 days. Cells were restimulated with DMSO or PMA (5 ng/ml) plus ionomycin (375 ng/ml) for 5h. Dot-plots show intracellular staining for IFN-γ versus IL-17.

Figure 4.1

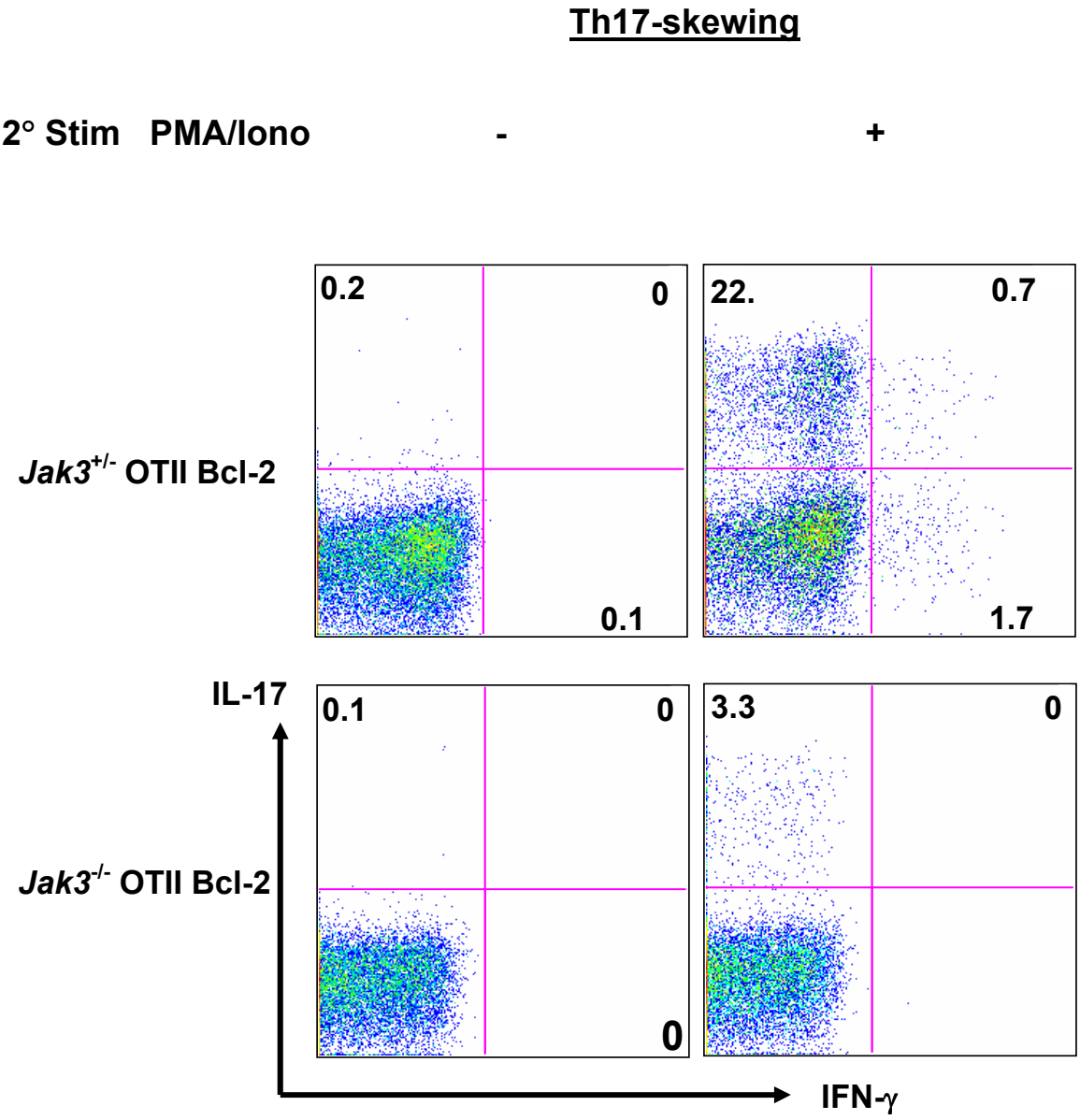
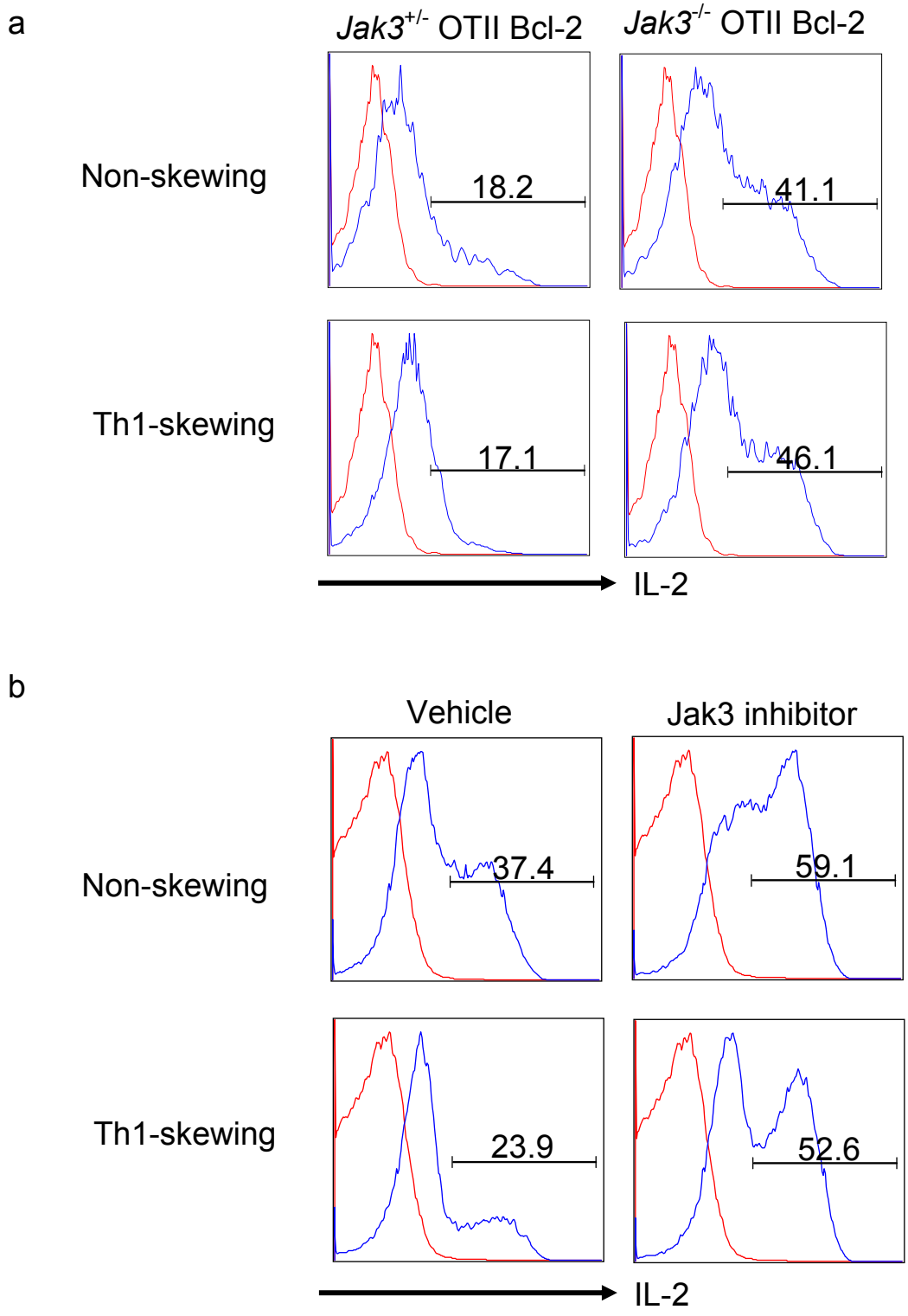

Th17-skewing
2° Stim PMA/Iono - +
Jak3+/- OTII Bcl-2
Jak3-/- OTII Bcl-2
0.2
0
0.1
22.
0.7
1.7
0.1
0
0
3.3
0
IL-17
IFN-γ

Figure 4.2 Jak3-deficient cells secrete more IL-2.

CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice (a) or CD4^{+} splenocytes from *Jak3*$^{+/-}$ OT-II-transgenic mice cultured with vehicle alone or PS078507 (625nM) (b), were stimulated under non- or Th1-skewing conditions for 4 days. Cells were restimulated with DMSO (red line) or PMA plus ionomycin (blue line) for 5 hours. Histograms show IL-2 intracellular staining.

Figure 4.2

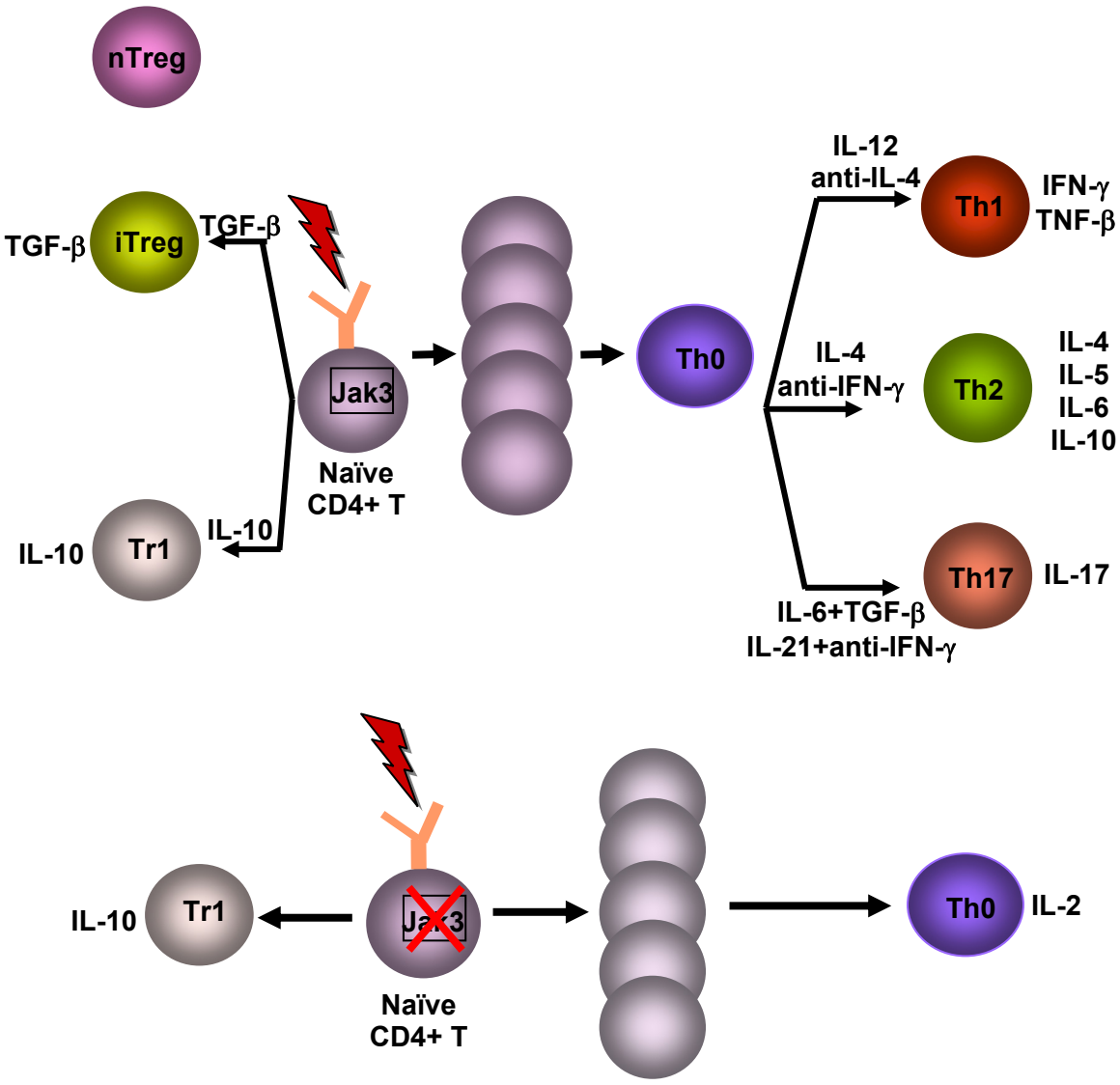

a
Jak3+/- OTII Bcl-2
Jak3-/- OTII Bcl-2
Non-skewing
18.2
41.1
Th1-skewing
17.1
46.1
IL-2
b
Vehicle
Jak3 inhibitor
Non-skewing
37.4
59.1
Th1-skewing
23.9
52.6
IL-2

Taken together, these studies suggest that in contrast to wild type $CD4^+$ T cells that differentiate into Th1, Th2 or Th17 cells after activation, T cells lacking Jak3-dependent cytokine signals either stay in the Th0 stage or differentiate into Tr1-like cells (Figure 4.3). Our work extends our understanding of the role of γc cytokines in adaptive immunity. Our data suggest that γc cytokines differentially regulate the proliferation and differentiation of naïve $CD4^+$ T cells, such that γc cytokines directly instruct naïve $CD4^+$ T cells to develop effector function without influencing cell cycle progression. Our study provides novel evidence that γc cytokines are key for naïve $CD4^+$ T cels differentiation into effector cells and that these cytokines are a crucial checkpoint for a productive cell response. Our data also indicate that γc cytokines control the generation of effector and regulatory T cells and regulate the fine balance between immune and autoimmune responses.

Figure 4.3 A model of $CD4^+$ T cell proliferation and differentiation in the absence of Jak3-dependent cytokine signals.

After antigen stimulation, naïve wild type $CD4^+$ T cells proliferate and differentiate into three major functional classes of T helper (Th) cells (Th1, Th2 and Th17) and several subsets of Treg cells (iTreg and Tr1), under the influence of specific cytokine environments. These subsets produce distinct cytokine profiles as indicated. Natural occurring Treg cells are generated from thymic T cell precusors. Although naïve Jak3-deficient $CD4^+$ T cells proliferate robustly to activation signals, they are not able to differentiate into Th1, Th2 or Th17 cells. Instead, Jak3-deficient cells either secrete a substantial amount of IL-2 and stay in the Th0 status or differentiate into Tr1-like cells.

Figure 4.3


nTreg
TGF-β
iTreg
TGF-β
Jak3
Naïve
CD4+ T
IL-10
Tr1
IL-10
Th0
IL-12
anti-IL-4
Th1
IFN-γ
TNF-β
IL-4
anti-IFN-γ
Th2
IL-4
IL-5
IL-6
IL-10
Th17
IL-17
IL-6+TGF-β
IL-21+anti-IFN-γ
IL-10
Tr1
Naïve
CD4+ T
Th0
IL-2

Signaling pathways for Th1 differentiation

The dogma concerning Th1 differentiation is that TCR ligation is a fundamental and initial step for Th1 differentiation (Figure 4.4). Signals from TCR engagement activate several transcription factors, including NFAT, which promote the basal level of IFN-γ production (55). Binding of IFN-γ to IFN-γR activates STAT1, which induces the expression of T-bet, the master transcription factor for Th1 differentiation (25). In addition to directly transactivating IFN-γ (27, 31), T-bet promotes IL-12Rβ2 chain expression to strengthen IL-12 signaling (23). Binding of IL-12 to IL-12R phosphorylates STAT4 to amplify Th1 responses. Our studies identified a novel pathway of Th1 differentiation, in which the IL-2/Jak3/STAT5 pathway controls the chromatin remodeling of the *Ifng* gene to permit the binding of T-bet to the *Ifng* promoter and transactivate gene expression.

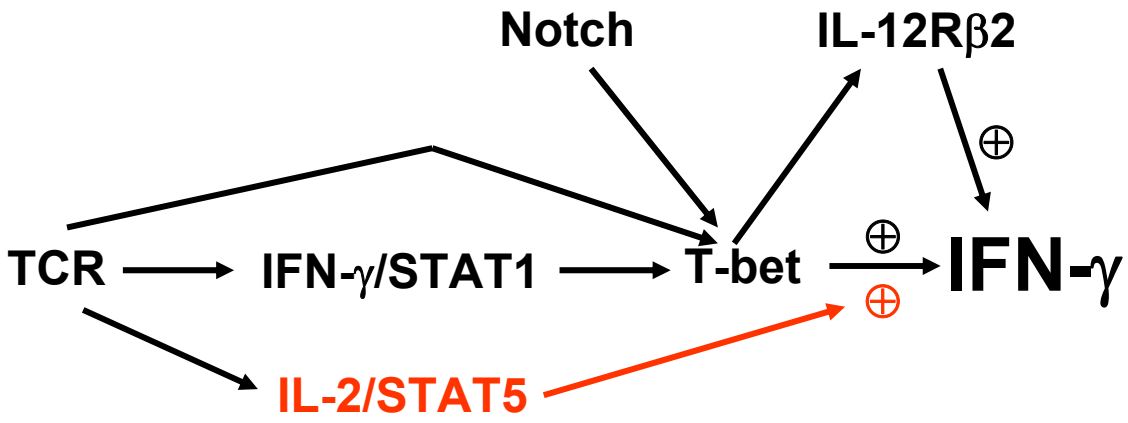
After TCR ligation, two parallel pathways are critical for T-bet function and Th1 differentiation. One is the IFN-γ/STAT1 pathway which regulates T-bet expression as mentioned above. The other is the IL-2/STAT5 pathway, which depends on the production of IL-2 and the expression of high affinity IL-2 receptor. The role of IL-2/Jak3/STAT5 signaling is to further open the *Ifng* locus, which is initially opened by TCR engagement, and to allow T-bet to bind to the *Ifng* promoter to transactivate the *Ifng* gene.

Alternativly, T-bet induction may not solely depend on the IFN-γ/STAT1 pathway. This idea is supported by several pieces of evidence. First, in our studies, we found that IFN-γR$^{-/-}$ T cells were still able to up-regulate T-bet and produce IFN-γ (data not shown). Second, kinetic studies showed that IFN-γR and STAT1 phosphorylation were down-regualted in the early stages of Th1 differentiation. For instance, IFN-γR was absent at 6h, 24h, 7d or 14d following Th1 culture (Figure 3.12, (204)) and STAT1 phosphorylation was also undetected at 3h or 24h after initial activation (Figure 3.12, (205)). This data indicate an althernative pathway to the induction of T-bet. The possible signals include TCR (209) and Notch signaling (210), which have been shown to induce T-bet expression. Nevertheless, the binding of T-bet to the *Ifng* promoter is controlled by the ability of IL-2/Jak3/STAT5 signaling to induce chromatin remodeling activity at the *Ifng* locus.

Figure 4.4 Signal pathways for IFN-γ transcription during Th1 differentiation.

TCR ligation promotes the basal level of IFN-γ production, which activates STAT1 through IFN-γR. Activated STAT1 induces T-bet expression. Alternatively, T-bet expression can be induced by TCR engagement or Notch signaling. In addition to directly transactivating IFN-γ, T-bet promotes IL-12Rβ2 chain expression to strengthen IL-12 signaling, which amplifies Th1 responses. The role of IL-2/Jak3/STAT5 signaling is to further open the *Ifng* locus, which is initially opened by TCR engagement, and to allow T-bet to bind to the *Ifng* promoter to transactivate the *Ifng* gene.

Figure 4.4



Molecular mechanisms for Th differentiation

Th subsets are classified by the distinct cytokine profiles which are the consequence of differential gene transcription. Gene transcription is regulated at multiple levels. One level of regulation is through controlling the abundance, modification and location of transcription factors. The other level is provided by epigenetic regulation of the accessibility of these transcription factors to their target elements. Thus, by modulating the probability with which transcription factors bind to the regulatory regions of genes, epigenetic regulation may act to set a threshold that transcription factors must exceed to promote gene expression.

The molecular mechanisms by which Jak3-dependent cytokine signals control the differentiation of naïve $CD4^+$ T cells is intriguing to explore. To date, several CNS sites have been found at the *Ifng* locus, located at 34kb, 22kb, 5kb and 0.3kb upstream and 18kb downstream of the transcription initiation site of the *Ifng* gene(90, 91, 95). These elements function as enhancers and are associated with Th1 cell specific expression of the *Ifng* gene. Among these, CNS-22 is unique due to its substantial histone modification, not only in Th1 cells but also in naive $CD4^+$ T cells. In this regard, it can be proposed that CNS-22 is a chromatin entry point for transcription or remodeling factors to expose other chromatin sites throughout the *Ifng* locus (91, 98). CNS-22 contains binding sites for a number of transcription factors, such as T-box factor, STAT, GATA, IRF, NF-κB and Ikaros, although which transcription or remodeling factor functions as a chromatin remodeling initiation factor is not clear. Our data demonstrate that Jak3/STAT5 is

involved in the epigenetic regulation of the *Ifng* locus and that STAT5 binds to the CNS-5 region during Th1 differentiation. It is tempting to speculate about a sequential model where other transcription factors and STAT5 function cooperatively to control the transcription of the *Ifng* gene. As shown in figure 4.5a, chromatin remodeling initiation factors bind to the CNS-22 element and initiate the chromatin remodeling of the *Ifng* gene, leading to the opening of the CNS-5 area to STAT5. Here, STAT5 acts as a checkpoint for further opening of the *Ifng* gene locus. Under the optimal Th1-skewing conditions, STAT5 further changes the chromatin configuration with the help of T-bet, resulting in the accessibility of other regulatory elements to remodeling factors. Subsequently, the proximal promoter of the *Ifng* gene is open to transcription factors, including T-bet, leading to the activation of *Ifng* gene transcription. The data presented in this thesis refine the understanding of Th1 differentiation by showing that in addition to general regulation, there is fine regulation of epigenetics and I propose that Jak3/STAT5 is a passport to fully open the *Ifng* gene locus. In addition to Th1 cells, CNS−22 appears to be essential for expression of the *Ifng* gene in $CD8^+$ T and NK cells (91). STAT5 has been found to bind to the *Ifng* locus in NK cells (199). Thus, the sequential model I proposed for Th1 cells may be applied to $CD8^+$ T and NK cells as well.

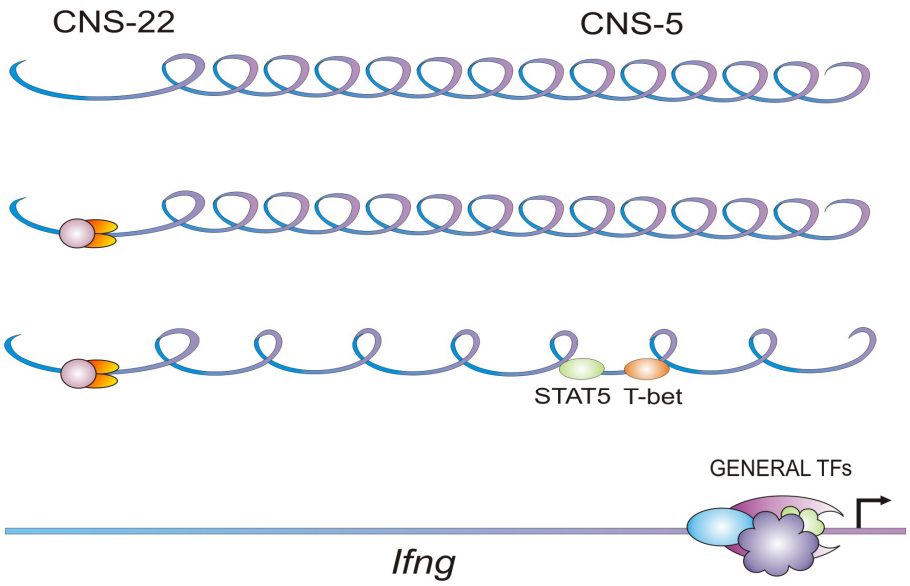
Figure 4.5 Model of the orderly chromatin changes in the *Ifng* and *Il4* locus during the commitment of naïve T cells to the Th1 or Th2 lineage.

(a) Chromatin of the *Ifng* gene in naïve $CD4^+$ T cells is basically condensed except the CNS-22 region. Chromatin initiation factors bind to CNS-22, leading to the partial opening of the *Ifng* locus and the exposure of CNS-5 region. STAT5 then accesses CNS-5 and fully opens the *Ifng* gene with the help of T-bet, which allows general and specific transcription factors to bind to the proximal promoter of the *Ifng* locus to activate *Ifng* gene transcription.

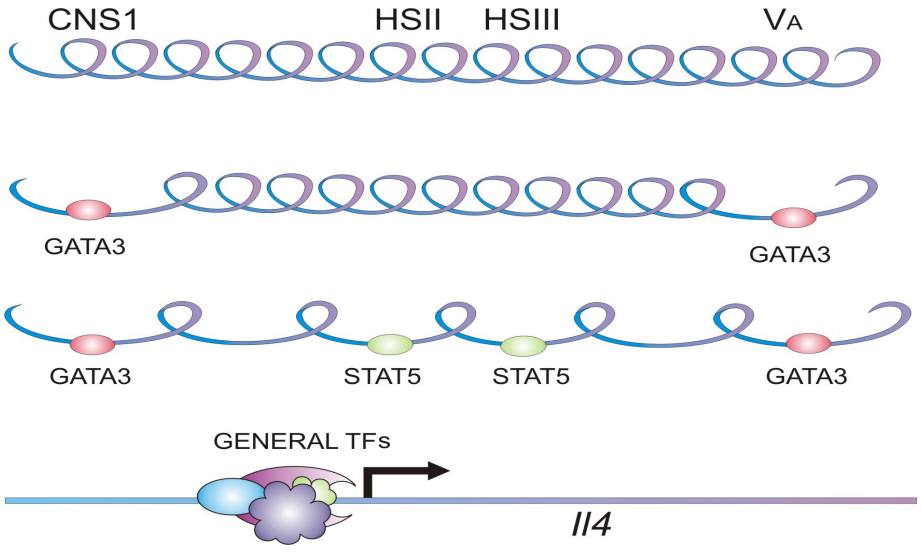
(b) In naïve $CD4^+$ T cells, chromatin structure along the *Il4* gene is highly compacted. Following activation, GATA3 binds to the regulatory element CNS1 and V_A, and mediates the partial remodeling of the *Il4* gene, resulting in the accessibility of HSII and HSIII sites. Binding of STAT5 to HSII and HSIII completely opens the *Il4* locus which allows for transcription factors to access the proximal promoter region and initiate *Il4* gene transcription.

Figure 4.5

a


CNS-22
CNS-5
STAT5
T-bet
GENERAL TFs
Ifng

b


CNS1
HSII
HSIII
VA
GATA3
GATA3
GATA3
STAT5
STAT5
GATA3
GENERAL TFs
Il4

Several regulatory elements have been identified in Th2 cells, which are DNase I hypersensitivity sites II and III (211), DNase I hypersensitivity sites 3′ of the *Il4* gene (HSV) (81), activation-dependent hypersensitivity site (V_A) (212)) and CNS1 (213) (Figure 4.5b). GATA3 has been shown to bind to CNS1 (214) (68) and V_A (68, 212) to open the *Il4* locus. Paul and colleagues demonstrate that STAT5, in contrast, binds to HS site II and III to remodel the *Il4* chromatin and lack of GATA3 or STAT5 leads to the incomplete opening of the *Il4* gene(73, 74). Furthermore, Paul's group reports that the initiation of chromatin remodeling around the HSII and HSIII region of the *Il4* locus is independent of IL-2/STAT5 signaling, whereas the maintenance of the accessibility of these regions needs IL-2/STAT5 signal (73). Based on these data, we speculate that GATA3 and STAT5 collaborate to regulate chromatin remodeling of the *Il4* gene, such that GATA3 binds to CNS1 and V_A to initiate chromatin remodeling of the *Il4* locus. Binding of STAT5 to HSII and HSIII provides permission to fully open the *Il4* gene locus. It is interesting to note that the mechanisms by which the lineage-specific and non-specific factors cooperate to render the accessibility of a lineage-specific gene are very similar between Th1 and Th2 cell differentiation.

Th17 cells are a newly discovered subset of Th cells that produce IL-17. It has been shown that STAT5-deficient cells produce more IL-17, suggesting that IL-2/STAT5 signaling constrains the generation of Th17 cells (215). However, the data presented in this thesis show that Jak3-deficient cells are unable to secrete IL-17 under Th17-skewing conditions. The disagreement of the Th17 cell generation between STAT5- and Jak3-

deficient cells could be explained as follows: STAT3 is an essential transcription factor for Th17 differentiation by directly promoting IL-17 production(216) or by inducing the expression of the Th17-specific transcription factor retinoic acid-related orphan receptor (RORγt) (21, 22). The activation of STAT3 may be reduced in Jak3-deficient cells because IL-21 is a γc cytokine that activates STAT3. This γc cytokine is crucial in amplifying Th17 differentiation and preferentially induces STAT3 and STAT1 activation through Jak3 and Jak1 (217). Therefore, cells lacking Jak3-dependent cytokine signals cannot differentiate into Th17 cells. In contrast, STAT5 suppresses IL-17 production through its competitive inhibition of STAT3 (215). Without the inhibitory effect of STAT5 during Th17 differentiation, STAT3 induces STAT5-deficient cells to produce more IL-17. The precise manner in which STAT3 functions in the Th17 generation remains to be elucidated. One possibility is that STAT3 regulates the expression of the *Il17a/f* gene at the chromatin level since STAT3 directly binds to the *Il17a/f* gene (216), and the polarization of Th17 cells is presumably accompanied by selective chromatin remodeling events (218). Therefore, it is possible that like Th1 and Th2 differentiation, Jak3-dependent cytokine signals regulate Th17 differentiation through epigenetic modification. Future work needs to be done to clarify whether STAT3 activation and RORγt expression are impaired without Jak3-dependent cytokine signals and how γc cytokine signals regulate the epigenetics of *Il17* locus.

IL-2 functions as a survival and differentiation factor for $CD4^+$ T cells following initial activation

Activation, proliferation and differentiation of T cells form the basis for the generation of immunocompetent cells, which allow the immune system to eliminate pathogens while protecting self structures. At the early stages of an immune response, also known as the expansion phase, activation of naïve T cells through the TCR and co-stimulatory molecules leads to the proliferation of antigen-specific T cells, which are needed to clear pathogens from the body. However, T cell proliferation is always accompanied by death to restrict the over-reaction of the immune responses. The outcome of T cell expansion depends on the net result of these two events. Each of these two processes may be under the control of multiple factors, such as TCR signaling, co-stimulatory triggering and signals from cytokines. Activation and proliferation of T cells result in their differentiation into distinct effector subsets depending on the cytokines present during the activation process.

IL-2 is a cytokine produced mainly by activated T lymphocytes. Kinetic studies show that the expression of IL-2 and the high affinity IL-2R (CD25) are in a transient manner. For example, IL-2 protein is detected several hours after antigen stimulation, peaks at 48h, and dramatically decrease at 72h (219, 220). Similar to IL-2 production, activated T cells express CD25 on their surface on day 1 after DC priming *in vivo* and down-regulate its expression to the level of naïve cells on day 3 (221, 222). Together, these data suggest that IL-2 functions in the early stages of immune responses.

As presented in chapter II, our data showed that T cell *in vitro* proliferation does not need signals from γc cytokines. Several studies have reported that γc cytokines are redundant for antigen-specific T cell proliferation *in vivo* (125, 130). At this point, we think that IL-2 may not directly affect the proliferation of T cells. TCR and co-stimulatory molecules provide strong signals to sufficiently drive cells into the cell cycle. However, our results suggest that the survival of activated T cell is greatly dependent on γc cytokines, which is supported by other studies (130). Using genechip analysis, we found that several anti-apoptotic factors are substantially down-regulated in activated $CD4^+$ T cells when IL-2 signals are blocked (unpublished data from our lab), such as Bcl-2, Bcl-3 and Pim-1 (Proviral integration of the moloney murine leukemia virus in mice). Furthermore, *in vivo* IL-2 therapy during the expansion phase enhances the magnitude of effector T cell responses (223, 224). These data indicate that IL-2 is critical for the survival of activated T cells after initial activation.

As shown in this thesis, IL-2 is essential for Th1 differentiation. It has been found that IL-2 is critical for Th2 differentiation (74, 215) and inhibits Th17 differentiation (215). Together, in addition to acting as a survival factor, IL-2 controls the $CD4^+$ T cell fate decision with the help of other lineage specific cytokines during the early stages of the immune responses.

Overall, upon encounter with antigen-carrying dendritic cells in the secondary lymphoid organs, naïve $CD4^+$ T cells become activated and proliferate, which is driven by TCR and co-stimulatory signals. In the first 3 days, activated T cells produce IL-2 and express CD25 for IL-2 responsiveness. The importance of IL-2 during this period is to maintain the survival of activated T cells and induce $CD4^+$ T cell differentiation.

The significance of this work

The work presented in this thesis attempts to understand the role of γc cytokine signals during T cell activation, proliferation, survival and differentiation. Studies of individual γc cytokines or γc cytokine receptors are important to establish the non-redundant roles of each cytokine. However, studies of Jak3, the master tyrosine kinase which transmits signals for all γc cytokines, have the unique capacity to discover additional and redundant functions of this family of cytokines.

Severe combined immunodeficienty (SCID) is a group of inherited defects in the immune system that has been recognized for more than 50 years. SCID patients fail to develop T or NK cells due to defective cytokine signaling. Although B cells are present in these patients, their function is greatly impaired. The defects in immune cell development and function lead to significant deficits in host defense (225, 226). Due to the recurrent and opportunistic infections with a potentially lethal outcome, SCID necessitates prompt diagnosis and treatment. It is known that mutation of *Jak3* gene is highly associated with

SCID (227, 228). Therefore, analysis of the animal model that lacks the function of Jak3 provides great insights into the pathophysiology of these disorders and offers new avenues for diagnosis and therapy of SCID patients**.**

Significant efforts are currently underway to develop pharmacological inhibitors of Jak3 for use as an immunosuppressive drug to inhibit autoimmune diseases, inflammatory diseases or organ graft rejection (229-231). A more thorough understanding of how these autoreactive or alloreactive T cells are generated and how this process contributes to the generation of effector T cells during an immune response may benefit the development of new strategies to block disease processes. Furthermore, knowing that agents used in disease treatment may have a number of side effects, a better knowledge of their mechanism of action may lead to the design of new molecules to improve the specificity.

CHAPTER V

Materials and Methods

Mice

Jak3$^{-/-}$ mice (112) were backcrossed to C57BL/6 for ten generations. OTII-transgenic (195)and Bcl2-transgenic (196) mice were purchased from Jackson laboratory (Bar Harbor, ME). *Jak3*$^{-/-}$ OT-II Bcl2 were generated by crossing *Jak3*$^{-/-}$ mice to OT-II and Bcl2-transgenic mice to generate *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OTII+ Bcl-2-transgenic mice. *Stat5ab* double-deficient mice (201) and CD2-cre transgenic mice (202) were generously provided by Dr. Joonsoo Kang with the permission of Dr. Lothar Hennighausen and Dr. Dmitris Kioussis, respectively. All mice and their littermate controls were sacrificed when they were 6-10 weeks of age. Mice were maintained at the University of Massachusetts Medical School specific pathogen-free animal facility after review and approval by the institutional animal care and use committee.

Jak3 inhibitor

PS078507 was developed at Pharmacopeia, Inc. (Princeton, NJ). A stock solution (10 mM) was prepared by dissolving PS078507 in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO). All working compound solutions were made by serial dilution in buffer or culture medium. DMSO was used as a vehicle control.

Kinase assays

Recombinant human Jak1 and Tyk2 were purchased from Carna Biosciences (Kobe, Japan). Recombinant human Jak3 and Jak2 were expressed using the Bac-to-Bac

Baculovirus Expression Systems according to the manufacturer's instructions (Invitrogen, Carlbad, CA). Briefly, cDNA fragments of Jak3 (508aa to 1124aa) and Jak2 (532aa to 1132aa) were amplified by PCR and cloned into the pFastBac vector with glutathione-S-transferase as a tag at the amino terminal to generate bacmid DNA. The Sf9 insect cells were used to generate baculovirus stock and to express recombinant Jak3 and Jak2. A homogeneous time-resolved fluorescence assay was used to assess kinase activity of Jak kinases using biotinylated poly Glu-Ala-Tyr as the protein substrate. Jak kinases were incubated with the substrate and ATP in the presence or absence of PS078507 for 60 min at room temperature. The reaction was terminated by adding EDTA, and the tyrosine phosphorylation was detected using Europium (LANCE) labeled anti-phosphotyrosine antibody (Eu-PT66; PerkinElmer, Wellesley, MA) and DyLight conjugated streptavidin (PIERCE, Rockford, IL). Time-resolved fluorescence signals were measured using a Victor^{2}V plate reader (PerkinElmer) at 615 nm and 665 nm.

Cell proliferation assay

Aliquots of cryopreserved peripheral blood mononuclear cells (PBMC) were purchased from AllCells (Emeryville, CA). PBMC were resuspended in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate). PBMC were cultured with the mitogenic lectin phytohemagluttinin (PHA) at a final concentration of 10 μg/ml for 3 days to upregulate IL-2 receptor and JAK3. PHA-activated PBMC were harvested, washed once with complete RPMI medium, resuspended in complete RPMI

medium, and seeded in 96-well plates (90 μl/well, 10,000 cells/well) in the presence or absence of IL-2 (10 ng/ml final; R&D Systems, Minneapolis, MN). Test compounds were serially diluted in DMSO followed by intermediate dilution in complete medium. Compounds (10 μl/well) were added to cells at a final DMSO concentration of 1%. Cells were incubated for 48 h at 37°C, 5% CO2. The number of viable cells was determined by the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Cell isolation, culture, and in vitro T cell differentiation

Thymocytes were harvested from $Jak3^{+/-}$ and $Jak3^{-/-}$ OT-II Bcl2 mice, red blood cells were lysed, and cells were incubated with anti-CD4-PE (BD Pharmingen, San Diego, CA) and anti-CD8-APC (BD Pharmingen). CD4 SP thymocytes were sorted by flow cytometry on a Mo-Flo sorter (Cytomation, Fort Collins, CO) to a purity of >98%. Splenocytes from $Jak3^{+/+}$ OTII-transgenic mice were incubated with CD4 antibody-coated magnetic microbeads, and $CD4^+$ T cells were purified by positive selection (Miltenyi, Auburn, CA) to a purity of >94%. T Cells were stimulated with plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) (eBioscience, San Diego, CA), unless the concentrations were specified. T cells were cultured in 24-well plates at 1.2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 iu/ml penicillin, 100 ug/ml streptomycin, 50 μg/ml geneticin, 2 uM β-mercaptoethanol, and 25 mM HEPES. For Th1 differentiation, cells were stimulated with

rIL-12 (5ng/ml) (R&D, Minneapolis, MN) and anti-IL-4 (5μg/ml) (BD Pharmingen). For Th2 differentiation, cells were stimulated with rIL-4 (20ng/ml) (R&D) and anti-IFN-γ (5μg/ml) (BD Pharmingen). For non-skewing conditions, no exogenous cytokines or antibodies were added. For all culture conditions, no exogenous IL-2 was added.

Analysis of cell proliferation by thymidine incorporation

Isolated $CD4^+$ $CD44^{lo}$ cells were plated out at a density of 1×10^5 cells /180 μl in triplicate in a 96-well flat-bottomed plate and stimulated with various concentrations of anti-CD3 and anti-CD28 or mitomycin C-treated C57BL/6 antigen presenting cells (APCs) plus the indicated concentrations of $OVA_{323-339}$. Forty-eight hours later, cells were pulsed with one microcurie of [3H]-thymidine for 18h, and harvested on a Tomtec Harvester 96 (Orange, CT). Thymidine incorporation was quantified on a Trilux microbeta counter (PerkinElmer, Wellesley, MA).

Analysis of cell proliferation with carboxyfluorescein diacetate succinimidyl ester (CFSE)

Isolated cells were washed once in PBS and resuspended at a density of 2.5×10^7 cells/ml in PBS. CFSE was added to a final concentration of 2.5 μM. The cell suspension was mixed thoroughly and placed at 37°C for 12 min and the reaction was terminated by adding RPMI 1640 with 10% fetal calf serum. The cells were then plated at 1.2×10^6 cells/ml in 24-well plates coated with anti-CD3 (1 μg/ml) and anti-CD28 (4 μg/ml)

antibodies. Three days later, the fluorescence of the cells was determined by flow cytometry.

Apoptotic analysis

Stimulated T cells were harvested, washed with cold PBS and resuspended in 1× BD Pharmingen™ Annexin V Binding Buffer to achieve a final concentration of 10.0×10^6 cells/ml. 5 μl of Annexin V-FITC and 7-AAD were added to 100 μl solution (~1×10^6 cells) and incubated at room temperature for 15 min in the dark. Apoptosis was analyzed by flow cytometry within 1h.

Cell cycle analysis by flow cytometry

One million naïve or activated T cells were washed with cold PBS and fixed overnight at -20°C in 95% ethanol. Cells were then pelleted, washed and resuspended in 1 ml PBS with the propidium iodide (PI) at a final concentration of 20 μg/ml, ribonuclease at 20 μg/ml and EDTA at 2mM. The cells were incubated at 37°C for 30 min in the dark. PI content were assessed by flow cytometry.

Intracellular cytokine staining

One million T cells cultured under non-skewing or Th1-skewing conditions were restimulated with phorbol myristate acetate (PMA) (5ng/ml) plus ionomycin (375ng/ml)

for 5h in a 96-well plate. Golgi Plug (MD Pharmingen) was added for the last 4h. The cells were first stained with cell surface marker, and then fixed, permeabilized, and stained with anti- IFN-γ-APC (BD Pharmingen) using intracellular staining Cytofix/Cytoperm kit protocol (BD Pharmingen), following the manufacture instructions.

Cytokine ELISA

Three hundred thousand T cells cultured under Th1- or Th2-polarizing conditions were restimulated with PMA (5ng/ml) plus ionomycin (375ng/ml) for 24h. Supernatants were harvested, serially diluted and assayed for IFN-γ, IL-4, IL-5 and IL-10 using cytokine detection kits (BD Pharmingen).

Real-time quantitative PCR

CD4 SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated for 4 days with anti-CD3 and anti-CD28 under non-, Th1- or Th2-skewing conditions. Freshly isolated CD4 SP thymocytes were used as controls. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Following DNAase treatment (Promega, Madison, WI), RNA was reversed transcribed into cDNA using Superscript II and Random Hexamers (Invitrogen), according to the manufacture instructions. Real-time quantitative PCR was performed on a Bio-Tad iCycler^{TM} by using SYBR® Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA). 18srRNA were used as internal control. All PCR reactions comprised 40

cycles of denaturation at 95°C for 25-30s, 25-35s of primer annealing at 58°C, 58.5°C, 62°C, 55°C, 56°C or 57.5°C for *Ifng, T-bet, 18srRNA, IL-12Rβ2*, *Ets-1* and *Hlx* respectively, and followed by a 25-30s extension step at 72°C. The following primers were used: *Ifng* sense, 5'-CCTGCAGAGCCAGATTATCTC-3', anti-sense, 5'-CCTTTTTCGCCTTGCTGTTGC-3'; *T-bet* sense, 5'-TTCCCATTCCTGTCCTTCACC-3', anti-sense, 5'-TGCCTTCTGCCTTTCCACAC-3'; *18srRNA* sense, 5'-TGGTGGAGGGATTTGTCTGG-3', anti-sense, 5'-TCAATCTCGGGTGGCTGAAC-3'; *IL-12Rβ2* sense, 5'-CCCAAGGAAATGAAAGGGAAT-3', anti-sense, 5'-TAGCGATGCAAATGCTTGATATC-3'; *Ets-1* sense, 5'-GCGCTACGTATACCGCTTTG-3', anti-sense, 5'-CAACCAACAGGGTTGCTCTT-3'; *Hlx* sense, 5'-GGGACAGTTCTTCGCATCTC-3', anti-sense, 5'-CTGTGGCATGGTGTCCTTAG-3'.

Immunoblot

Naïve cells or activated cells were harvested at the indicated time points, washed and lysed in RIPA buffer for 20 min on ice. The protein fraction was separated by centrifugation at 13,000 rpm for 10 min at 4°C and protein level was quantified with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were immunoblotted with antibodies to cyclin D2 (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin E (Santa Cruz Biotechnology), cyclin A (Santa Cruz Biotechnology), p27kip1 (Santa Cruz

Biotechnology), PI-3-kinase p85 (Cell Signaling Technology, Danvers, MA), phospho-Stat5 (Cell Signaling Technology) and β-actin (BD Pharmingen) antibodies.

Freshly isolated cells (naïve) or Th1-polarizing cells (Th1) were harvested, lysates were prepared, proteins were separated by PAGE, and transferred to nylon membranes. Membranes were immunoblotted with antibodies to T-bet (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (BD Pharmingen), phospho-Stat1 (Tyr701) (Cell Signaling Technology, Danvers, MA), total Stat1 (Cell Signaling Technology), phospho-Stat4 (Y693) (Invitrogen, Carlsbad,CA), total Stat4 (Santa Cruz Biotechnology) and phospho-Stat5 (Cell Signaling Technology) antibodies.

Chromatin immunoprecipitation

ChIP assays were performed by using the ChIP Assay Kit (Upstate Cell signaling Solutions, Charlottesville, VA), according to manufacture's instructions with minor modifications. Briefly, 1.5×10^6 cells were fixed by adding formaldehyde directly to culture medium to a final concentration of 1% and incubated at 37°C for 10 minutes. After incubation, glycine was added to a final concentration of 0.125M for 5 minutes. Cells were rinsed once with ice cold PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml aprotinin and 1μg/ml pepstatin A). Cells pellets were resuspended in 100μl of SDS Lysis Buffer containing protease inhibitors indicated as above and incubated for 10 minutes on ice. The suspension was sonicated with ultrasonic processor (cole-parmer instrument, Vernon Hills, Illinois) for 6 times with

at least 1 minute cooling on ice in-between. The conditions for each time as follows: amplitude 40, timer 1min, and pulser 10sec. Cellular debris was removed by centrifugation for 10 minutes at 13,000g at 4°C. The supernatant was diluted 10 fold in ChIP Dilution Buffer with protease inhibitor as above. An aliquot of diluted supernatant was used as input DNA control after reverse cross-linking. The diluted cell supernatant was pre-cleared with agitation with Protein A Agarose/ Salmon Sperm DNA for 1 hour at 4°C. Agarose was discarded by briefly centrifugation. Immunoprecipitation overnight at 4°C on a rotor was performed by using anti-T-bet (H-210, Santa Cruz), anti-acetylated histone H3 (Upstate), and anti-Stat5a (R&D), with anti-IgG (Santa Cruz) as a negative control. Immunocomplexes were precipitated with Protein A Agarose/ Salmon Sperm DNA on a rotor for 1 hour at 4°C, washed 5 times with provided Wash Buffers and eluted with freshly prepared elution buffer (1%SDS, 0.1M NaHCO3). Following reverse cross-linking and protein digestion, the DNA was recovered by phenol-chloroform extraction, precipitated with the help of 20 μg of glycogen and resuspended in 50 μl of TE buffer.

PCR was performed with 5μl from a total of 50μl of the immunoprecipitated DNA with various primers. As a control, the PCR was done directly on input DNA purified from chromatin before immunoprecipitation. PCR products were resolved on 2.5% agarose gels or 6% polyacrylamide gels and visualized with ethidium bromide staining or silver staining, respectively. For T-bet ChIP assay, PCR comprised 40 cycles (of denaturation at 95°C for 30s, primer annealing at 56°C for 30s and lastly a 30s extension step at 72°C.

For Histone H3 ChIP assay, PCRs included 30 cycles of denaturation at 95°C for 30s, 30s of primer annealing at 60°C or 55°C for primer set #3 and #4 respectively, followed by an extension step at 72°C for 30s. For Stat5a ChIP assay, PCRs were performed for 40 cycles of denaturation at 95°C for 30s, 30s of primer annealing at 56°C and 30s of extension at 72°C. Positions of primers are indicated on a map of the murine *Ifng* locus (Figure 2.6). Primers to detect T-bet binding (Lovett-Racke et al., 2004) were set #1 (Figure2.6): 5'-CACGTTGACCCTGAGTGAT-3' and 5'- GAGGAAACTCTTGGGCTTC-3'; primers for Stat5a binding were set #6 for *Ifng*CNS-34: 5'-AAGCCCAGAGTGTCAACCAC-3' and 5'-GGATCATCTAGCAGCCGTTG-3', set #7 for *Ifng*CNS-22: 5'-CACAGGAAGGAGATGGGAAG-3' and 5'-CCCGTTAACCTTCTGCTCTG-3', set # 8 for *Ifng*CNS-5.5: 5'-CCGTGTGGACTTCCATTCTC-3' and 5'-GTTACCTCCTCCACCCGTTC-3', set #9 for HS-3.6: 5'-ATCAGGAAGGAACAGGCTTC-3' and 5'-CCATCCTTTCGTCTCAGCTC-3', set #5 for HS-0.3: 5'-TGCTGTGCTCTGTGGATGAG-3' and 5'-GGGCTCTCTGACGATGAGAC-3', and set #10 for *Ifng* CNS+18: 5'-AAGCCAGTTTGTCATCATGC-3' and 5'-TTTGTGCTTTCCTGATTCACC-3'; primers to examine Histone H3 acetylation were set #3, 5'-CGTAATCCCGAGGAGCCTC-3' and 5'-CTTTCAATGACTGTGCCGTGG-3', and set #4, 5'-GCTCTGTGGATGAGAAAT-3' and 5'-AAGATGGTGACAGAAGG-3'.

Restriction enzyme accessibility assay

Restriction enzyme accessibility experiments using LM-PCR were performed as previously described (de la Serna et al., 2005) with some modifications. CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl2 mice were stimulated under Th1-skewing conditions for 4 days. Freshly-isolated cells (naïve) were used as controls. 6×10^6 Cells from each group were washed with cold PBS, resuspended in 1ml of freshly prepared lysis buffer (25mM Hepes at pH7.9, 15mM MgCl2, 10mM KCl, 0.1% NP40, 1mM DTT, 1mM phenylmethylsulfonyl fluoride PMSF, 1μg/ml aprotinin and 1μg/ml pepstatin A) and incubated on ice for 10 minutes. Cells were homogenized by 20 strokes with Dounce B Homogenizer. Nuclei were spanned down at 1,000g for 4 minutes, washed once with lysis buffer and resuspended in 100 μl of digestion buffer (300mM sucrose, 1× restriction enzyme digestion buffer and 100 μg/ml BSA). 14 μg nuclei were digested with 14U or 28U HinfI at room temperature for 90 minutes with periodic agitation. The digestion was stopped by adding an equal volume of stop buffer (20mM Tris at pH 7.4, 20mM EDTA, 0.4% SDS, 300mM NaCl and 1mg/ml proteinase K) and incubating overnight at 50°C. Genomic DNA was purified by Phenol-Chloroform extraction, precipitated by ethanol, resuspended in TE and the DNA concentration was determined by 260nm absorbance. HinfI LM-PCR adaptors were generated by annealing the two oligos (5'-AATGAATTCAGATC-3' and 5'-GCGGTGACCCGGGAGATCTGAATTC-3') at 95°C for 5 min followed by cooling to 25°C. Adaptor ligation was performed by using DNA Ligation Kit Ver.2.2 (TaKaRa), according to manufacturer's instruction. Basically, 1 μg of genomic DNA was mixed with 1 μl of 100μM adaptor, water was then added to bring

the volume to 5 μl. After adding 5 μl of Solution II (DNA Ligatin Kit Ver.2.2, TaKaRa) and 10 μl of Solution I (DNA Ligatin Kit Ver.2.2 TaKaRa), the ligation reaction was incubated overnight at 16°C. T4 DNA ligase was inactivated by heating the reaction at 70°C for 10 minutes. LM-PCR comprised 35 cycles (30s at 95°C, 30s at 60°C and 30s at 72°C). PCR for input control included 35 cycles (30s at 95°C, 30s at 56°C and 30s at 72°C). The primers for LM-PCR (Figure 2.6, primer set #2) were as follows: 5'-GGATATTACCACCAAAACTACGCAG-3' (on *Ifng* promoter) and 5'-GGTGACCCGGGAGATCTGAATTCATTCG-3' (on LM-PCR adaptor). Input DNA was monitored by amplifying the *Ifng* promoter in a region that cannot be digested by HinfI using the following primers: 5'-GTTGACCGTGGTTGATGTTG-3', and 5'-TTGTACCTTGGACCTATACTATGC-3'.

Statistical Analysis

Statistical analysis was performed using the two-tailed paired student's t test.

CHAPTER VI

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